

# FUNDAMENTALS OF Microbiology

SIXTH EDITION, ILLUSTRATED

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## Preface to the 6th Edition

THE AIM in this edition has been to present current information about fundamental principles of microbiology, with modern exemplifications, in a simple, direct and concise form, yet, having in mind the burdens of the college student of today, in as readable and interesting a manner as possible. Because of enormous expansion of research in the field, exhaustive coverage is no longer feasible in an elementary textbook. For this reason the material included has been very carefully selected. It is intended for the student interested in acquiring an introduction to the field of microbiology; whether for future professional specialization or as part of a well-rounded general education—for the Liberal Arts student, students in Home Economics, Pharmacy, Agriculture, Industry, Premedical and Veterinary courses. It is assumed that the reader has at least an elementary knowledge of chemistry, physics and biology.

This edition represents almost a complete rewriting. The chemistry and physics of metabolism and enzymes have been much simplified. The chapters on viruses have been completely rewritten in line with new developments, both in concept and technique. So, also, have the sections on growth, structure, methodology, mutation, variation and sex, antibiotics, disinfection, microbiology of soil, of water and sewage, of industrial spoilage, the lactic acid bacteria, the anaerobes and numerous others. The emphasis on infectious diseases and public health, while still present in appropriate aspects, has been lessened and more has been placed on other important aspects of microbiology. Much information has been tabulated for conciseness and the convenience of the student.

A special effort has been made to improve the illustrative material and to make the explanatory legends carry as much of a story as possible. Scientists at home and abroad have contributed generously of superb electron micrography and microphotography and the author feels a special gratitude to them. All are thankfully acknowledged, as well as the courtesy of publishers, in legends accompanying the pictures.

It is neither practicable nor desirable to include complete bibliographies. The references listed after each chapter have been selected with care as to content, date and inclusive bibliography as well as to pertinence. Recent review articles have been included so far as possible. It is hoped these lists may afford keys to the literature for the interested student and useful material for instructors in fields other than their specialties.

It is a pleasure to acknowledge the informed direction of Dr. Maurice

Green, Assistant Professor of Microbiology, St. Louis University, in the preparation of Chapter 13 (Nutrition, Metabolism and Cultivation of Microorganisms) and the wise counsel of Dr. Libero Ajello and of Dr. Lucille Georg, both of the Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia, in revising Chapters 3 and 4 (Yeasts and Molds). Their efforts are evident in the good there is in these sections; the author is responsible for any defects.

Finally, only the expert guidance of the publisher's staff and the unflagging assistance of the author's long-suffering helpmate, Amy W. Frobisher, have made possible the transformation of rough manuscript into a beautifully crafted volume. To both the author expresses his undying thankfulness.

MARTIN FROBISHER, JR.

*May, 1957*

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## SECTION 1

# *The Relationships of Microorganisms to Each Other and to the Living World*

THE FIELD of microbiology is a large one. There are many species, genera, tribes, orders and classes of microorganisms. One may devote one's life to any of a vast number of highly specialized problems. Some of these sound utterly fantastic: the role and exploitation of certain protozoa in the purification of sewage; how to make poultry and livestock out of sawdust; what microscopic fungi interest the telephone companies and why; how to find (and manufacture?) petroleum with bacteria; how to control the fertility of the soil; development of an antibiotic, an antimetabolite or a viral vaccine to combat cancer—and so on. Before becoming an expert in any one of these always fascinating and sometimes lucrative activities one must have a good basic idea of what microorganisms are: their form, structure and size; what they do; how they act; how they are controlled, classified, identified; and several other useful facts about them. The first section of this textbook introduces the beginning student to the eight great biological groups in which microorganisms occur: algae, protozoa, yeasts, molds, bacteria, viruses, rickettsiae and pleuropneumonia-like organisms. In this section each group is described in sufficient detail, and in comparison with the other groups, so that the beginning student may define and distinguish each and identify any microorganism with respect to its major group. This section opens the door, as it were, to that beckoning enigma—the living microscopic universe.



# The Microscopic World

## 1. PROTOZOA, ALGAE, FUNGI AND BACTERIA

THE WORLD of microorganisms must not be thought of as sharply differentiated from the world of macroscopic life. If we begin our survey of life with the largest of living things, such as whales or giant redwood trees, and move downward in the scale of size to those of less and less magnitude, it immediately becomes clear that there is a continuous gradation in size, from the very large to the very small. As we proceed downward in the scale we reach a point where, because of diminishing size, the objects of our survey disappear, as it were, behind a curtain representing the limit of our unaided eyes to see (*resolve*) minute objects. They have entered the realm of the microscopic. We may follow with our eyes into the microscopic world for a short distance by poking a hole through the curtain with a microscope. On the other side of the curtain we find, as did Alice when she passed "Through the Looking Glass," many more strange and beautiful creatures. In a still-diminishing scale of size these extend far back into the secret shadows of the seeable cosmos to some inconceivably small vanishing point among the molecules, atoms, electrons, neutrons, protons, and the unknown.

**Animal or Vegetable?** We generally think of the living world as divided into two well-defined kingdoms: animal and vegetable. Usually in the macroscopic world the properties of plants distinguish them clearly from animals. One could never confuse a rabbit with a rose. But when one cannot see the creatures he wishes to distinguish, when one cannot even accurately determine form, color, chemical properties, food, mode of multiplication, waste products, and other matters of vital importance to the classifier, how is he to know in which kingdom to place them, especially when he finds creatures possessing some of the properties of both plants and animals? This problem has led some workers to suggest a third kingdom, the *Protista*, to include all microscopic creatures not readily classified as plant or animal. This suggestion has been discussed but never generally adopted.

The places of creatures which are pretty clearly plants, and of those which have definitely animal characters, are well established in their respective kingdoms, as shown in the accompanying diagram (Fig. 1-1). In the diagram it is

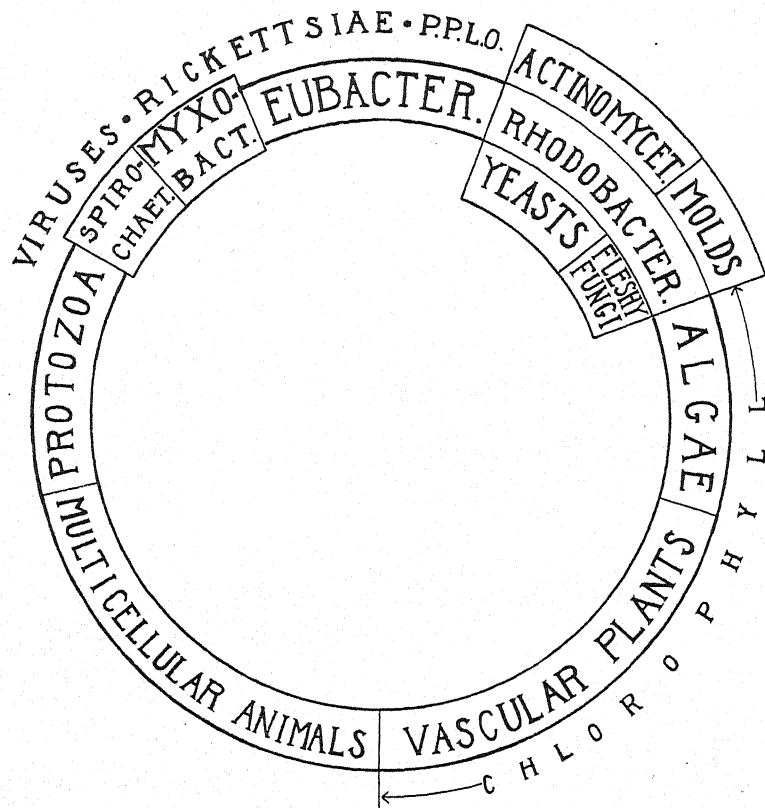


Fig. 1-1. A suggested relationship of various divisions of animate nature. With a few borderline exceptions microscopic forms are found in all groups except multicellular animals and vascular plants. Of the bacteria, Spirochaetales are shown as most like protozoa; Myxobacteriales are perhaps more like spirochaetes than they are like other bacteria because of their flexing movements. The Eubacteriales (Eubacteriineae) have least resemblance to protozoa or algae. The Rhodobacteriineae are seen as most like the algae because of their photosynthetic pigment. True chlorophyll is shown only for algae and vascular plants. The Actinomycetales are most like the molds, while yeasts, perhaps, are more like true bacteria than are the molds although certainly this arrangement is open to debate. The position of the Chlamydobacteriales is doubtful and is not shown. Probably it should be next to Rhodobacteriineae since the Chlamydobacteriales resemble algae in some ways but are without photosynthetic pigment. The relationships of the viruses, rickettsiae and pleuropneumonia-like organisms (P.P.L.O.) are entirely speculative.

seen that the field of the microbiologist embraces one phylum of the animal kingdom (Protozoa—microscopic animals) and parts of one phylum of the vegetable kingdom (Thallophyta—microscopic fungi and some minute algae). Some of the Schizomycetes, or bacteria, such as the Spirochaetales, closely resemble protozoa. Others, such as the Actinomycetales, have some interesting resemblances to the molds. Still others (Rhodobacteriineae) have some fundamental properties (photosynthesis) of the algae. Although lines are drawn to make convenient diagrammatic distinctions between these groups, the distinctions in nature are far from clear. There are many puzzling overlappings

and morphological and physiological resemblances between apparently distant groups.

In Figure 1-1 three groups of microscopic organisms are shown outside the animal-vegetable circle: the viruses, the rickettsiae and the pleuropneumonia-like organisms (PPLO). Although some of these have certain properties of some bacteria, they defy accurate classification in either animal or vegetable kingdom.

In order to orient the beginner in this *Alice-in-Wonderland* universe of the microscopic, it is desirable to describe briefly the principal characteristics of each of the eight groups with which we shall deal (protozoa, algae, bacteria, viruses, rickettsiae, pleuropneumonia organisms, yeast and molds), and to show how the groups are interrelated, and their place in the living world as a whole. These properties are summarized in Table 1.

### BACTERIA AND THE ANIMAL KINGDOM

If we examine the animal kingdom as a whole, we find the vertebrates, with man at the top of the list, representing the latest and most complex development in organic evolution. Proceeding downward we pass through groups of lower animals, such as the Arthropoda, Annelida, Rotifera, and Porifera, each less and less highly developed functionally and structurally, and finally we come to the protozoa, the least complex of all animals and the most primitive in an evolutionary sense. Only among the protozoa do we find any forms in the least resembling any of the bacteria, and this resemblance is not confusing except, perhaps, among certain spirochetes.

**Bacteria and Protozoa.** In water in which a bit of hay has been allowed to soak for a few days, or in sewage, not only do many kinds of bacteria abound, but also various fantastic and elegant creatures which resemble bacteria in some respects but which can, as a rule, be readily differentiated by their relatively huge size, their elliptical or ovoid form and other distinctive features. These are protozoa (Fig. 1-2)—microscopic animals, each consisting of only a single cell.

Protozoa vary greatly in size, according to species and physiological state. Most of them are hundreds of times as large as most bacteria. A commonly studied type, *Paramecium*, is roughly elliptical in shape and has dimensions of about  $200\mu$  by  $40\mu$ .\* Protozoa are the most primitive members of the animal kingdom, just as bacteria are the lowliest group in the vegetable kingdom. As contrasted with bacteria structurally, the protozoa are very complex and usually contain well-defined portions performing the functions of specialized organs in more highly organized animals. Bacteria do not contain such *organelles*, except flagella.

There are thousands of species of protozoa, and they may be found in almost every habitable situation on the earth; stagnant water, pond mud, surface waters, feces, the soil, dust, the ocean and so on. They live, in part, upon other minute living things, including bacteria, and most of them also take in soluble foods by diffusion through the cell membrane. A few are pathogenic.

The protozoa seem to have evolved in the direction of complexity of physi-

\*  $\mu$  is the symbol used for micron, the unit of length commonly employed in microbiology. A micron is 0.001 millimeter or about 1/25,400 inch. The dimensions of *Escherichia coli*, a cylindrically shaped bacterium common in sewage, vary around  $1\mu$  by  $10\mu$ .



**Table 1. Characteristics of Microorganisms and Higher Animal Cells**

ALGAE	PROTOZOA	MOLDS AND YEAST	BACTERIA	PLEUROPNEUMONIA- LIKE ORGANISMS	RICKETTSIAE	VIRUSES	HUMAN CANCER AND OTHER ANIMAL TISSUE CELLS
<ol style="list-style-type: none"> <li>Require sunlight; grow on lifeless media.</li> <li>Multiply by fission and sexually.</li> <li>Much larger than bacteria.</li> <li>Do not pass filters.</li> <li>Cell structure like higher plants.*</li> <li>Cannot ingest solid particles.</li> </ol>	<ol style="list-style-type: none"> <li>Grow in dark; some on lifeless media.</li> <li>Multiply by budding, conidia, and sexually.</li> <li>Size much greater than bacteria — filaments and other structures often microscopic.</li> <li>Do not pass filters.</li> <li>Cell structure, especially nuclei, visible.</li> <li>Cannot ingest solid particles.</li> </ol>	<ol style="list-style-type: none"> <li>Grow in dark on lifeless media.</li> <li>Multiply by fission mainly.</li> <li>Round forms do not exceed about 5 microns, usually not over 2 to 3 microns in diameter; rod forms usually not over 2 microns in diameter and 10 microns in length. Some species of spirochetes may exceed 500 microns in length but not over 1 to 2 microns diameter. Do not pass filters (except spirochetes).</li> <li>No definite organs in the cell visible with ordinary microscopes.</li> <li>Granules, spores, capsules.</li> <li>Cannot ingest solid particles.</li> </ol>	<ol style="list-style-type: none"> <li>Grow in dark on lifeless media.</li> <li>Complex life cycle.</li> <li>Very minute, except spherical "large bodies." Pass bacterial filters.</li> <li>No definite internal structure.</li> <li>Mode of nutrition unknown; presumably like bacteria.</li> </ol>	<ol style="list-style-type: none"> <li>Grow only in living cells.</li> <li>Method of multiplication unknown.</li> <li>Probably fission.</li> <li>Much smaller than bacteria. Have rod, coccus, and spiral forms.</li> <li>Some pass bacterial filters.</li> <li>Internally like bacterium-like (?).</li> <li>Mode of nutrition unknown; presumably like bacteria.</li> </ol>	<ol style="list-style-type: none"> <li>Grow only in living cells.</li> <li>Method of multiplication unknown.</li> <li>Not visible by ordinary means. By electron microscope some appear to have a spherical, cuboidal or "tadpole" form.</li> <li>Pass bacterial filters.</li> <li>No definite structures visible.</li> <li>Mode of nutrition unknown.</li> </ol>	<ol style="list-style-type: none"> <li>Grown in dark on lifeless media.</li> <li>Multiply by fission.</li> <li>Much larger than bacteria. Do not pass filters.</li> <li>Complex internal structure with nucleus, nucleolus, etc.</li> <li>Cannot ingest solid particles (except phagocytic cells).</li> </ol>	

\* Except blue-green algae which have bacterium-like nucleus.

ological function without increase in number of cells. Thus, in many free-living protozoa a portion of the cell is somewhat thickened, rigid and sometimes chitinous\* in composition. This serves as a skin or outer integument. It often contains (or forms) bristles (cilia or flagella) which have the functions of swimming appendages of higher animals. Although there are no proper muscles, certain portions of the protoplasm may be contractile and may act the part of muscles in moving the cilia or flagella. The integument excretes waste products much as do the skin and kidneys of a human being, and thus serves the function not only of swimming oars, muscle and skeleton, but also of kidney, intestine and sweat glands. In so far the protozoa and bacteria have much in common. Inside the cell membrane of protozoa, however, there are usually a well-defined nucleus, nucleolus and cytoplasm, the last often containing various vacuoles which collect waste products eventually to be ex-

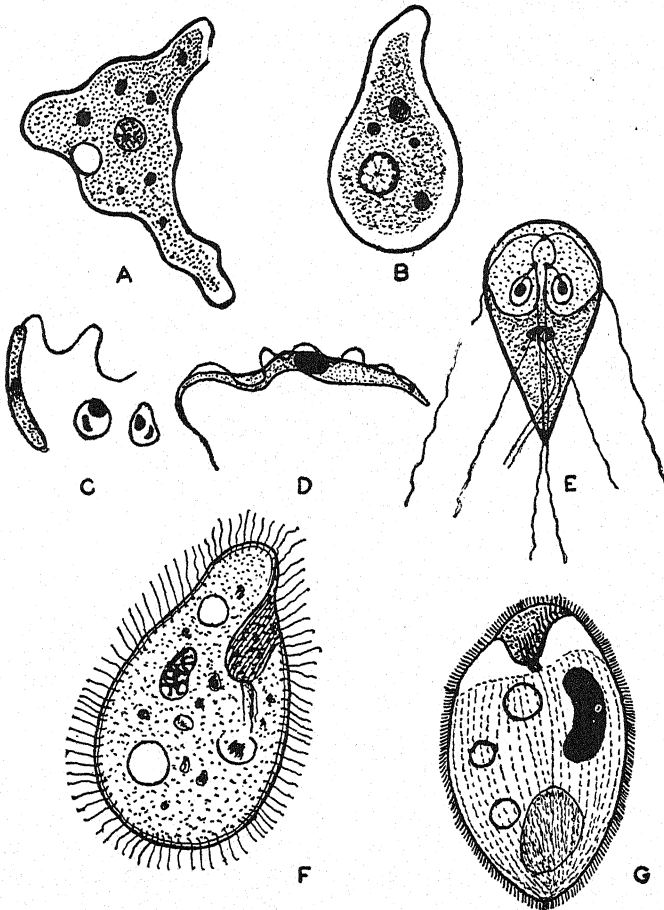


Fig. 1-2. Various forms of protozoa. A, B, forms of amoeba. C, D, forms of trypanosome. E, *Giardia lamblia*. F, G, types of ciliates. (After Hegner, Cort and Root, from Burdon, "Medical Microbiology," by permission of The Macmillan Co., publishers.)

\* Chitin is a rigid skeletal substance common in the animal kingdom and is represented by the shells or exoskeletons of insects, lobsters, etc.

creted through the cell wall, thus serving the purpose of bladder and colon of higher animals. Other vacuoles contain food particles in the process of digestion and thus are analogous to stomachs. There is often an opening which serves as a mouth and gullet. Bacteria contain none of these. In protozoa there are also granules of stored-up reserve material equivalent to fatty deposits or liver-glycogen reserves in mammals. Bacteria often store up such granules of food reserve.

Although no such complex structures as nose, eyes or nerves are found in protozoa, many protozoa are nevertheless quite sensitive to heat, chemicals, gravity, electricity and light; and in one species at least (*Euglena*), a portion of the cell is differentiated into a specially sensitive area called a "light spot." This, although not a separate organ, serves the function of a primitive eye. There are also some bacteria which appear to possess a light-sensitive spot or zone (see *Rhodobacteriineae*). Furthermore, protozoa in general accept or reject food particles or waste, and have quite a delicate sense of touch so that they recoil on contact with hard objects, turning aside quite as though they were highly sensate and responsive creatures; all of which shows the marvellous possibilities contained within a single, microscopic droplet of protoplasm. Reproductive processes are, in some protozoa, similar to those observed in the higher animals, inasmuch as there are sexual phenomena, with conjugation, nuclear interminglings, etc., in all essential respects analogous to the sexual phenomena of fertilization in higher plants and animals. However, there are usually no special sexual organs. There are no *well-defined* sexual phenomena in bacteria, though this is much debated.

The cells of all living creatures possess the power of simple, sexless, or *asexual* multiplication. It is spoken of as *cell fission*. In the unicellular forms, as in bacteria and protozoa, each cell resulting from fission remains a separate organism. In complex forms, different cells group themselves together as into "guilds" and tissues (the organs of the body, for example), such as the liver, the brain, the heart. In the single-celled organism each cell fission leads to the formation of two entire, complete, new individuals; in many-celled organisms cell fission generally adds only to the size of the individual of which the cell is a part.

**Classification of Protozoa.** A complete classification of the protozoa would be beyond the scope of this book. A brief outline of the main groups will, however, be useful in later discussion. All of the protozoa which we shall discuss are motile during at least one stage of their existence, the means of motility being different in each group. These differences are used as a basis of classification. There are four great groups of protozoa and one or more species in each group is pathogenic. The groups may be listed, with one or two common examples, as follows:

CLASS I. *Sarcodina* (move with pseudopods\*).

A. *Endamoeba histolytica*—causes amoebiasis.

B. *Endamoeba coli*—not pathogenic.

CLASS II. *Mastigophora* (move with long, whip-like lashes called flagella).

A. Intestinal tract:

(a) *Giardia lamblia*—possibly causes enteritis.

(b) *Trichomonas hominis*—not pathogenic.

\* Irregular, lobular or finger-like projections constantly being thrust out from, or retracted into, the cell at any part of the cell surface.

- B. Urogenital tract:
  - (a) *Trichomonas foetus*—a cause of abortion in cattle.
  - (b) *Trichomonas vaginalis*—may cause vaginitis in women.
- C. Blood stream:
  - (a) *Trypanosoma rhodesiense*—causes African sleeping sickness.
  - (b) *Trypanosoma gambiense*—causes African sleeping sickness.
  - (c) *Trypanosoma equiperdum*—causes dourine ("equine syphilis") of horses.
  - (d) *Trypanosoma cruzi*—causes Chagas' disease.
- D. Blood stream and tissues:
  - (a) *Leishmania donovani*—causes kala-azar.
  - (b) *Leishmania tropica*—causes oriental sore.
  - (c) *Leishmania braziliensis*—causes espundia or South American leishmaniasis.

CLASS III. *Sporozoa* (move with pseudopods only in immature stages; male gamete is flagellate).

- A. *Plasmodium vivax* (also *P. malariae*, *P. falciparum* and *P. ovale*)—cause malaria.

CLASS IV. *Ciliata* (move with cilia).

- A. *Balantidium coli*—causes enteritis and ulcerations.
- B. *Paramecium caudatum*—not pathogenic.

Some authors include a fifth class, Suctoria, in which young stages are ciliated while adult stages are provided with tentacles.

**Reproduction and Life Cycles of Protozoa.** Some protozoa multiply both sexually and asexually, others only by *binary fission*. Cell fission is said to be binary when the cell divides into two approximately equal parts, each part having all of the physiological and genetic equipment of the parent cell.\* Unlike the bacteria, the flagellate protozoa divide lengthwise (longitudinal fission) rather than crosswise (transverse fission). Ciliate protozoa divide transversely. The nucleus undergoes changes very much like mitosis. Bacteria exhibit only transverse binary fission.

Many protozoa, during their lifetime, pass through a definite and readily demonstrable series of developmental stages and are thus quite different from the bacteria. These series of developmental stages are called *life cycles* and often are quite complicated. For example, in the case of the malarial parasite and some other protozoa, especially those which live in the blood, it is necessary for the parasite in its various stages to live in an insect host† before it is mature and ready to infect man or animal again. The cycle involving sexual reproduction usually takes place in one host (the definitive host) while the asexual development occurs in another (intermediate host). This phenomenon of the developmental cycles is spoken of as *alternation of generations*. In the case of the malarial parasite the definitive host is a certain kind of mosquito, while the intermediate host is man. In the trypanosomes the invertebrate hosts may be tsetse flies and other insects, the mammalian host depending on the species of parasite. Bacteria, with a few possible exceptions, exhibit no such complex life cycles, if any at all.

**Mode of Nutrition.** One property which distinguishes most plants (including bacteria) from most animals (including protozoa) is the manner of

\* Sometimes in binary fission the two daughter cells are not *exactly* alike.

† In speaking of parasites and disease-producing (pathogenic) microorganisms, the creature on which the parasite or pathogen lives is called the *host*, though usually an unwitting or unwilling one.

taking nourishment. There are some exceptions, like parasitic protozoa and worms, but typically, animals are able to take *solid masses* of food *into the body* (be it a many-celled or a single-celled body), there digesting it and turning it into soluble substances which nourish the cell or cells of the animal. This *holozoic* mode of nutrition is obvious enough in large animals, but may readily be observed in the smallest forms such as ameba or paramecium with the aid of a microscope.\* With a few exceptions, like "Venus's fly trap," the food of all plants, from the apple tree to the fern, seaweed, fungus or bacterium, is absorbed through the cell walls of root or thallus, and this absorption can occur only when the food is in the form of relatively *simple compounds soluble in water or cell contents*; plants cannot *ingest* solid food. Their mode of nutrition is *holophytic*.

### BACTERIA AND THE VEGETABLE KINGDOM

Let us now examine the vegetable kingdom as a whole and consider the different divisions and their relations to each other and to the animal kingdom, especially the protozoa. Opinions differ as to the most logical method of classifying the organisms of the vegetable kingdom and no system can be said to be the best for all purposes. For the present, the schema given below is a convenient arrangement.

#### THE VEGETABLE KINGDOM

**Phylum I.** Tracheophyta (vascular plants). Contain chlorophyll.†

*Subphylum 1.* Pteridopsida. Ferns and seed plants.

Class 1. Angiospermae or Spermatophyta (flowering plants).

Subclass 1. Monocotyledoneae (plants with 1 seed leaf)—lilies, grasses, etc.

Subclass 2. Dicotyledoneae (plants with 2 seed leaves)—roses, zinnias, maples, etc.

Class 2. Gymnospermae (naked seeds)—pines, etc.

Class 3. Filicineae—ferns.

*Subphylum 2.* Sphenopsida—horsetails.

*Subphylum 3.* Lycopsida—club mosses.

**Phylum II.** Bryophyta—Liverworts and true mosses. Contain chlorophyll.

Class 1. Musci—mosses—*Sphagnum*, *Polytrichum*, etc.

Class 2. Liverworts—*Marchantia*, etc.

**Phylum III.** Thallophyta—no roots, stems, leaves or flowers.

*Subphylum 1.* Algae—contain chlorophyll.

Class 1. Chlorophyceae—Green algae.

Class 2. Rhodophyceae—Red algae.

Class 3. Phaeophyceae—Brown algae (and Diatoms?).

Class 4. Myxophyceae—Blue-green algae.

*Subphylum 2.* Fungi—Do not contain chlorophyll.

Class 1. Basidiomycetes—mushrooms, puffballs, tree fungi, etc.

Class 2. Ascomycetes—yeasts, blue molds, mildews, etc.

Class 3. Phycomycetes—"bread molds," etc.

Class 4. Fungi imperfecti—heterogeneous asexual fungi of classes 2 and 3.

Class 5. Schizomycetes—fission fungi, bacteria.

Phyla I and II may be disposed of as including only macroscopic green plants of relatively complex structure and showing well-developed sexual differentiation. None of these morphologically resembles bacteria or protozoa.

\* If we consider the *tissue cells* of large animals, even this difference largely disappears, because the tissue cells, like bacteria and most plants, depend on food in solution.

† Chlorophyll is the green pigment of familiar plants like grass and trees. By means of chlorophyll the plants absorb the energy of sunlight and use that energy to synthesize the plant structures. The process is called *photosynthesis*.

Phylum III, subphylum 1, contains many plants of relatively simple structure. Flowers and seeds are unknown. In spite of the fact that some of them (as the kelp-algae or seaweeds of the Pacific) may attain very large size (*Macrocystis*—600 feet in length), none is possessed of any well-differentiated root, stem or leaf structure, although certain seaweeds approach this complexity.

**Bacteria and Algae.** As shown in Figure 1-1 the nearest relatives of the bacteria in the vegetable kingdom are the algae and the fungi. A brief description of algae and of fungi will show the principal similarities and differences.

Algae are mainly aquatic and marine plants, found in sunlit waters over a wide range of latitude. Like all thallophytes, they have no definite roots, vascular stem, flowers or seeds. Each plant consists of only one kind of tissue, which is distinctive for that species. Leafy, lacy and filamentous forms found on beaches, on shallow lake bottoms, the beds of streams, etc., are often called "sea lettuce," "seaweed," "pond or brook slime," and the like. Algae frequently also grow at the surface of stagnant, sunny ponds, forming a bright green scum. *Scenedesmus* is a good example. Rocks and trees in damp sunlit places in the woods frequently carry a green film of algae, and some species of algae grow on the surface of moist, fertile farm soils.

Algae are like other green plants in having cellulose (or cellulose-like) cell walls, and in using solar energy in photosynthesis. With minor exceptions, bacteria differ from algae in not having cellulose cell walls and in having no chlorophyll. Except for one small group, bacteria do not use solar energy but obtain energy from chemical reactions. They are independent of, and often killed by, sunlight.

Algae are important as sources of food for many fish and other forms of marine life dependent on fish; as sources of plant food in the soil; as a source of the jellylike *agar* used to solidify bacteriological culture media (seaweed *Gelidium*); and as nuisances when they grow in impounding reservoirs for city water supplies or in the private cistern. In the latter they may be controlled by exclusion of sunlight. In reservoirs they are often controlled by dissolving minute amounts of copper sulfate in the water. Algae have been cultivated artificially on a commercial scale and may become important sources of food for livestock and man in the future.

**THE CYANOPHYCEAE.** The algae most closely related to bacteria are the blue-green algae or Cyanophyceae (cyano=blue; phyceae=algae) or Myxophyceae. The name Myxophyceae is derived from a slimy capsule or outer envelope (myxo=slime). With a few exceptions, these algae contain green chloro-

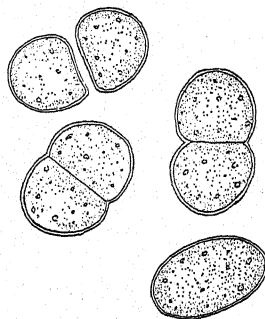


Fig. 1-3. *Synechococcus*, a blue-green alga the cells of which are solitary except during fission. Several stages of fission are shown as well as a single cell about ready for division. (Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," published by John Wiley & Sons, Inc.)

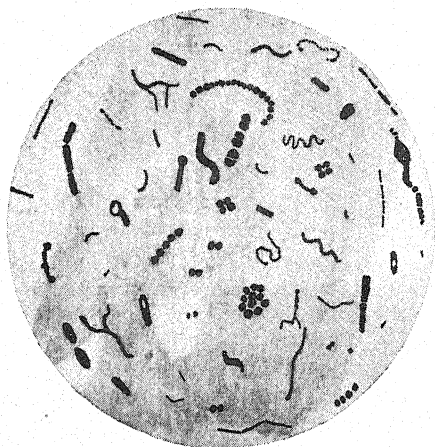


Fig. 1-4. Various forms of bacteria, magnified about 1000 times. These have been stained to make them more readily visible. Shown here are spherical cells (cocci) in chains, irregular clumps, pairs and groups of four; straight, rod-like cells (bacilli) variously shaped: some very thin, some thick, some with dormant enclosures (endospores); a few curved, rod-like cells (vibrios); some spiral and wavy cells (spirochetes); some branched cells (actinomycetes); and some odd-shaped, variant forms.

phyll and a blue pigment called phycocyanin (hence the name Cyanophyceae). These are the simplest, smallest and most bacteria-like of the sunlight-dependent plants. Good examples are *Synechococcus* (Fig. 1-3), *Gloeocapsa*, and *Oscillatoria*.

The cyanophyceae, like bacteria, multiply by binary fission and have no sexual cycle. They are coated by a slimy capsule or sheath.\* Their nucleus is indistinct and presumably consists of granules of nuclear matter diffused throughout the cell (a *diffuse nucleus*). Their chlorophyll is also diffuse, not confined to chloroplasts as it is in higher plants. The bacteriochlorophyll-containing bacteria (Rhodobacteriineae) are shown in Figure 1-1 as nearest to the algae.

**Bacteria and Fungi.** Turning our attention to the fungi, we are struck by the absence of chlorophyll from all.† Among the fungi are microscopic forms which do not resemble ordinary plants in size or appearance and which are indifferent to, or even killed by, sunlight. Among these are the yeasts, the molds, and the smallest and simplest of all of the fungi: the bacteria.

All fungi, including bacteria, lack the power of utilizing sunlight.† They make use of energy derived from chemical reactions to live and multiply. They synthesize their cell substances from soluble substances, using such *chemical energy*. Such synthetic processes are called *chemosynthesis*.

Among the large, fleshy fungi such as mushrooms, bracket fungi and puffballs, or the woolly molds seen on decaying organic matter in warm weather, and even among the microscopic cells of yeasts, there are rudiments of sexual differentiation.

**Recapitulation.** When we descend to the lowly bacteria we encounter fungi (a) generally devoid of chlorophyll and therefore chemosynthetic; (b) only 1 or 2 microns in diameter; (c) existing as relatively simple, single cells or as small, easily-broken-up agglomerations of independent cells; (d) devoid of readily demonstrable sex; (e) without definite nucleus; (f) multiplying mainly by transverse, binary fission and hence called fission fungi or Schizomycetes (*schizo* = fission; *mycetes* = fungi) (Fig. 1-4).

\* In many bacteria this is readily demonstrable, in others only with difficulty.

† With the exception of the Rhodobacteriineae, which contain bacteriochlorophyll.

## DEFINITION OF TERMS

Before proceeding further to compare bacteria with other forms of life we may describe briefly certain common properties of bacteria to which it is frequently necessary to refer in such comparisons.

**S  $\rightleftharpoons$  R Variation.** First is the smooth  $\rightleftharpoons$  rough (S  $\rightleftharpoons$  R) variation; two alternate forms in which most bacteria often appear (and which probably have their analogues in most other microorganisms).

The terms rough and smooth describe the appearance of colonies\* of bacteria. The surface of S colonies is smooth, often glistening or satiny. The margins of S colonies are even and regular. The cells in S colonies usually occur singly and are often covered with a coating of protective substance called a capsule or slime layer. They exhibit other distinctive properties to be described later. R colonies are rough, "crumbly," granular, rugose, irregular. The margins of the colonies are irregular, serrated, notched, scalloped. The cells usually occur in chains or clumps and are usually devoid of capsule and have other distinctive properties to be described (Figs. 1-5 and 1-6).

R and S forms are two of many kinds of variants which occur in many species of bacteria and possibly other organisms. Any bacterial species may produce either R or S form, or both at once.

**Aerobic and Anaerobic Growth.** A noteworthy property of many microorganisms (including bacteria) is their ability to thrive on lifeless media *in the total absence of air or free oxygen*. This astonishing fact was discovered by Pasteur in 1861. He referred to the phenomenon as anaerobiosis, or "life-without-air." Some are capable of normal life in either the presence or absence of air. Such are said to be "*facultative*" with respect to oxygen. Others are *restricted* to anaerobic conditions and are, indeed, killed by exposure to air or

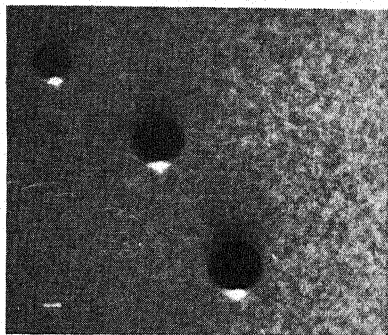


Fig. 1-5.

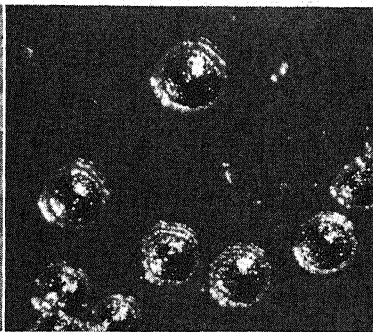


Fig. 1-6.

Fig. 1-5. Smooth colonies of gram-positive rod: same species as Fig. 1-6.

Fig. 1-6. Rough colonies of a gram-positive rod: same species as Fig. 1-5.

These colonies are growing on a jelly-like (agar) surface containing nutrient substances for the bacteria. Each colony contains billions of living bacterial cells. Compare the smooth surface and regular margins of the "S" colonies with the rough surfaces and irregular margins of the "R" colonies. These pictures are about five times actual size (indicated by the symbol  $\times 5$ ).

\* Colonies are small, visible masses of bacteria growing on a solid, nutrient surface (Fig. 36-5).



free oxygen. Such organisms are said to be *strict* or *obligate anaerobes*. Still other microorganisms can live only in the *presence* of air or free oxygen. These are called *obligate aerobes*. Some microorganisms thrive best in an environment of *partial* anaerobiosis or reduced oxygen tension. These are spoken of as *microaerophilic*. The biological significance and biochemical mechanisms of anaerobiosis and aerobiosis will be discussed later on.

These relationships may be summarized as follows:

#### RELATIONS OF MICROORGANISMS TO OXYGEN

##### I. Anaerobiosis

A. *Strict*—cannot grow in presence of *free* oxygen.

B. *Facultative*—can grow in presence or absence of free oxygen.

##### II. Aerobiosis

A. *Strict*—cannot grow in absence of free oxygen.

B. *Facultative*—see under anaerobiosis.

##### III. Microaerophilism

Grow best in reduced oxygen tension.

#### REFERENCES

- Alexopoulos, C. J.: *Introductory Mycology*. John Wiley and Sons, New York, 1952.
- Burrows, W.: *Textbook of Microbiology*, 16th ed. W. B. Saunders Co., Philadelphia, 1954.
- Christensen, C. M.: *The Molds and Man*. University of Minnesota Press, Minneapolis, 1951.
- Coulter, M. C.: *The Story of the Plant Kingdom*. University of Chicago Press, Chicago, 1935.
- Faure-Fremiet, E.: Morphology of protozoa. *Ann. Rev. Microb.*, 1953, 7:1.
- Faust, E. C.: *Animal Agents and Vectors of Disease*. Lea & Febiger, Philadelphia, 1955.
- Fogg, G. E.: *The Metabolism of Algae*. John Wiley and Sons, New York, 1953.
- Hegner, R. W.: *Big Fleas Have Little Fleas*. Williams & Wilkins Co., Baltimore, 1938.
- Hutner, S. H., Trager, W., and others: Growth of Protozoa. *Ann. New York Acad. Sci.*, 1953, 56:815.
- Hylander, C. J.: *The World of Plant Life*. 2nd ed. The MacMillan Co., New York, 1956.
- Jahn, T. L., and Jahn, F. F.: *How to Know the Protozoa*. Brown Publishing Co., Dubuque, Iowa, 1949.
- Kluyver, A. J., and van Niel, C. B.: *The Microbe's Contribution to Biology*. Harvard University Press, Cambridge, Mass., 1955.
- Lwoff, A.: *Biochemistry and Physiology of Protozoa*. Academic Press, Inc., New York, I, 1951; II, 1955.
- McBee, R. H., Lamanna, C., and Weeks, O. B.: Definitions of bacterial oxygen relationships. *Bact. Rev.*, 1955, 19:45.
- Milner, H. W.: Algae as food. *Sci. Amer.*, 1953, 189:31.
- Pringsheim, E. G.: The relationship between bacteria and myxophyceae. *Bact. Rev.*, 1949, 13:47.
- Rogers, J. S., Hubbell, T. H., and Byers, C. F.: *Man and the Biological World*. Rev. ed. University of Chicago Press, Chicago, 1952.
- Schwimmer, M.: *The Role of Algae and Plankton in Medicine*. Grune and Stratton, New York, 1955.
- Smith, G. M.: *Cryptogamic Botany. I. Algae and Fungi*, 2nd ed. McGraw-Hill Book Co., New York, 1955.
- Villee, C. A.: *Biology*, 3rd ed. W. B. Saunders Co., Philadelphia, 1957.
- Weatherwax, P.: *Botany*. 3rd ed. W. B. Saunders Co., Philadelphia, 1956.
- Weiss, F. J.: The useful algae. *Sci. Amer.*, 1952, 187:15.

# The Microscopic World

## 2. GROWTH OF BACTERIOLOGY

THE TERM bacteria as used today comprises about 1600 species of microscopic, unicellular fungi having a variety of biochemical and physiological properties and a relatively wide range in microscopic size and shape. Since about 1667, when bacteria were first observed, we have learned several things about them. For example, some are motile by means of flagella; others not. The individual cells of some species are spherical. Others are cylindrical, like a cigarette or sausage; some are spirally curved.

We now know that, although bacteria are extremely minute, measuring often about  $1/50,000$  of an inch ( $0.5 \mu$ ) in diameter and weighing as little as  $4/10,000,000,000,000$  (four ten-trillionths) of a gram, they are not unimportant and they do not experience undue difficulty in the struggle for existence. Persistence of a species does not depend on great size but upon the power to live and multiply under a variety of conditions.

Let us imagine creatures, for example, which can function in a range of temperatures from freezing to almost boiling, and regardless of whether free oxygen and food, as we know it, be present or not; creatures which, in addition to actively carrying on the business of life under these circumstances, can double their numbers every twenty minutes or, if required, cease growth and go into a state of what seems to be completely suspended animation, in this state surviving cold so intense as to liquefy hydrogen, extreme drought prolonged for many years, heat so intense as to coagulate blood or "hard boil" an egg, high pressures and high vacua. Let us imagine, furthermore, that the creatures are so minute as to be invisible to any enemies possessed of eyes with which to hunt them. Such creatures, it is clear, would be far more likely to survive the rigors of environmental change, and competition with larger forms of life, than unwieldy and relatively vulnerable structures like men, animals and trees. Such gifted organisms are to be found among the bacteria and related microorganisms. Some of them are admirably adapted to survive under conditions such as those described, some of which probably existed in the early geological history of the earth; and to survive all the known succeeding vicissitudes of climatic change since those periods to the



Fig. 2-1. The world before life. (Field Museum of Natural History.)

exclusion, if need be, of other, more advanced, forms of life. It seems not unlikely that, unless some grand cataclysm destroy all living things suddenly, such bacteria may be the last survivors of the pageant of life on this globe.

**The Primitive World.** Bacteria are primitive and it may be supposed that they represent the type of living beings which appeared very early in the earth's history of life. Indeed, bacteria have been regarded as the first cellular inhabitants of this planet. Probably, however, creatures much smaller, and similar to *genes* or *viruses*, preceded bacteria in the evolutionary procession.

The bacteria of primeval times probably lived in the sea or mud, and from them probably evolved many of the microorganisms familiar to us today. Familiarity with microorganisms, however, is of rather recent achievement since they could be studied accurately only after the development of lenses of sufficient magnifying power to make them visible. The role of the microscope in microbiology is, therefore, important.

**The First Microscopes. Leeuwenhoek.** By the end of the seventeenth century lenses had already been exploited in various ways for many years, Roger Bacon having used them experimentally before 1294. Such lenses, however, did not magnify very highly. The man mainly responsible for revealing the whole, hitherto unknown and unseen world of microorganisms was the Dutch investigator (Fig. 2-2), *Antoni van Leeuwenhoek* (1632-1723), a linen merchant by trade and influential in civic affairs as well. Leeuwenhoek was an active, intelligent man of public and commercial affairs in the city of Delft. He was not a trained scientist but was self-educated and of rare intellectual powers. He amused himself by means of his skill and craftsmanship in glass blowing, fine metal work and other occupations. He came of a well-to-do family and lived in relatively easy circumstances with plenty of leisure for his avocation of making minute but powerful lenses. With these he delighted in examining a great variety of objects. He examined saliva, pepper decoctions, cork, the leaves of plants, circulating blood in the tail of a salamander, seminal fluid, urine, cow dung, scrapings from the teeth and so on. In many of these he saw living creatures, some of which we now know were protozoa and bacteria but all of which he called "animalcules."

In spite of the fact that his microscopes were not compound he obtained

remarkable results with them. "... he showed rare ingenuity and expert craftsmanship in the grinding and mounting of his simple lenses, a skill which he zealously kept to himself; and in spite of the requests of his learned friends, he refused to disclose the secret of his success." "... Leeuwenhoek's instruments are not true microscopes at all in the sense in which we think of microscopes, but rather simple magnifying glasses generally consisting of a small, single, biconvex lens. The object, and not the lens, was moved into focus by means of screws" (Fig. 2-3). "To adjust the lens to the object was so long and tedious a task that it is not surprising that Leeuwenhoek used an individual lens for each object. . . ." "The magnification varied and at best did not exceed two hundred to three hundred diameters." "The size of objects which Leeuwenhoek examined was determined by comparison. For this purpose he used at various times a grain of sand, the seed of millet or mustard, the eye of a louse, a vinegar eel, and still later hair or blood corpuscles. In this way he secured fairly accurate measurements of a great variety of objects." "... he was forced to admit that the sand grain was more than one million times the size of one of the animalcules."

Leeuwenhoek was so interested in the things he observed that he wrote minutely detailed reports about them to the Royal Society in London. His first letter was dated in 1674. He was later elected a Fellow of the Royal Society. Some of his observations are at once quaint and epoch-making. For example, after examining material which he scraped from between his teeth, he said, "Though my teeth are kept usually very clean, nevertheless when I view them in a Magnifying Glass, I find growing between them a little white

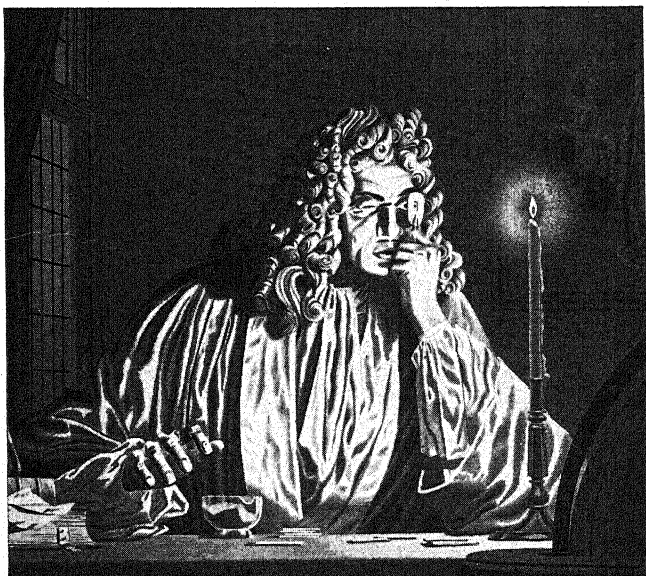


Fig. 2-2. Antonj van Leeuwenhoek. A fanciful delineation based on a famous portrait. The picture shows accurately the size and shape of the first microscopes, the manner in which they were used, and the simple laboratory apparatus of the "Father of Bacteriology." (Courtesy of Lambert Pharmacal Co.)

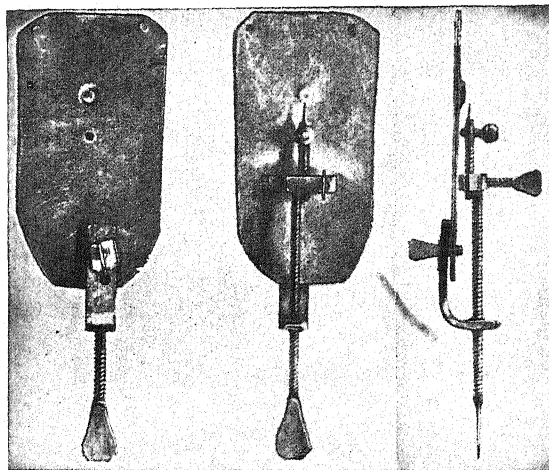


Fig. 2-3. One of Leeuwenhoek's microscopes: side, back and front views.

The tiny spherical or hemispherical lens is held in the slightly raised structure in the upper part of the metal plate. The object to be examined was mounted at the tip of the sharp-pointed mounting pin. Focussing was accomplished by means of the three thumb screws to which the mounting pin is attached. These are approximately actual size.

matter as thick as wetted flour; in this substance, though I could not perceive any motion, I judged there might probably be living Creatures.

"I therefore took some of this flour and mixt it either with pure rain water wherein were no Animals; or else with some of my Spittle (having no Air bubbles to cause a motion in it) and then to my great surprise perceived that the aforesaid matter contained very small living animals, which moved themselves very extravagantly. The biggest sort had the shape of A (Fig. 2-4). Their motion was strong and nimble, and they darted themselves through the water or spittle, as a Jack or Pike does through the water. These were generally not many in number. The second sort had the shape of B. These spun about like a top, or took a course sometimes on one side, as is shown at C and D. They were more in number than the first. In the third sort I could not well distinguish the Figure, for sometimes it seem'd to be an Oval, and other times a Circle. These were so small they seem'd no bigger than E and therewithal so swift, that I can compare them to nothing better than a swarm of Flies or Gnats, flying and turning among one another in a small space." (E. B. Fred.)

When microorganisms were thus first brought within the range of human vision, they were viewed by students of natural philosophy from various standpoints. Some considered them the original and lowest forms of life; others hailed them as the cause of disease. Many debates arose, some of which still go on. Those concerning the origin of bacteria and their relation to the problem of the origin of life were especially vigorous and make interesting reading. It is desirable to review some of these, since a number of discoveries were made, during the experimental studies on the subject, which are important in present-day microbiology. Without a knowledge of these earlier discoveries, the student of microbiology works at a disadvantage.

**Ancient Theories Concerning the Origin of Life.** The ancients knew nothing of microorganisms, or of evolution or of the fact that only living things could beget living things. They believed in spontaneous generation, i.e., that creatures like frogs, mice, bees and other animals sprang fully-formed from fertile mud, decaying carcasses, warm rain or fog, and the like. *Van Helmont* (1577-1644) devised a method for *manufacturing mice*. He recommended putting some wheat grains with soiled linen and cheese into an appropriate receptacle and leaving it undisturbed for a time in an attic or stable. Mice would then appear. This observation may still be *experimentally confirmed* but the conclusions drawn from it differ today. But belief in spontaneous generation lived on for years.

For example, an elderly lady of the writer's early acquaintance complained bitterly that she had been cheated by a merchant who sold her a woolen coat which was of such a quality that it turned entirely into moths when left undisturbed in a closet for some months!

Such empirical theories of spontaneous generation of living beings were later discarded; but only after a most dramatic intellectual and experimental struggle, which will be detailed presently. All such theories assumed that life begins as fully-formed creatures of complex structure. It was later realized that life must have originated in much simpler forms.

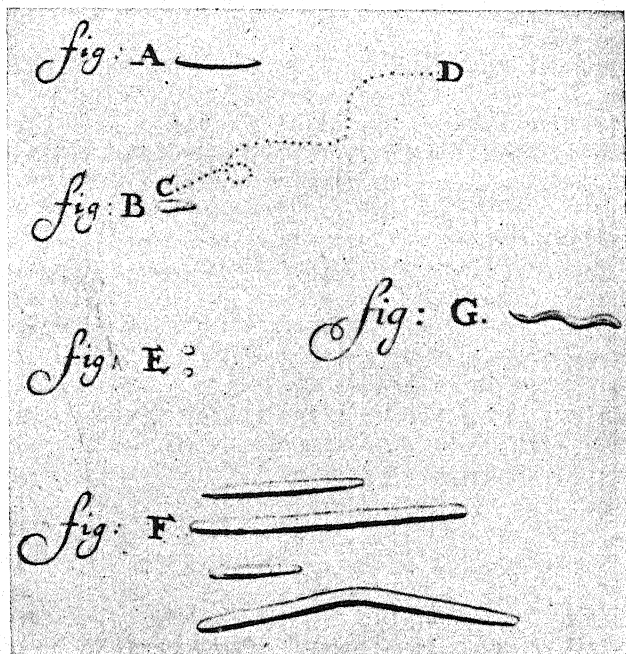


Fig. 2-4. Leeuwenhoek's drawings of bacteria.

Here may be seen cocci, bacilli and (probably) a spirochete. The motion of one of the bacilli is clearly indicated. Today such observations are commonplace. But Leeuwenhoek was seeing them for the first time in the history of the Human Race! It was as momentous a discovery as that of Columbus—a New World!

## THE DISPUTE OVER SPONTANEOUS GENERATION

In the earlier years, in the absence of exact knowledge of microorganisms or chemistry, there had already arisen much skepticism and bitter feeling over the question of the origin of life. One "scientist" who still held to the ancient ideas says of the views of another, who doubted, "So may we doubt whether, in cheese and timber, worms are generated, or if beetles and wasps in cow dung, or if butterflies, locusts, shell-fish, snails, eels, and such life be procreated of putrefied matter which is to receive the forms of that creature to which it is by formative power disposed. To question this is to question reason, sense and experience. If he doubts this let him go to Egypt and there he will find the fields swarming with mice begot of the mud of Nylus, to the great calamity of the inhabitants." There was a great deal of such acrid discussion by wordy savants of the times who tried to settle everything by argument. Experimentation was regarded as rather undignified and even smacking of relations with The Evil One.\*

**Francesco Redi (1626-1679).** The experimental method was, however, being invoked here and there. For example, it had always been supposed that the maggots in decaying meat were derived spontaneously from transformations of the putrid meat itself. Redi, a physician of Arezzo, questioned this and placed meat and fish in jars covered with very fine gauze. He saw flies approach the jars and crawl on the gauze. He saw the eggs of the flies caught on the gauze and observed that the meat then putrefied without maggot formation. Maggots developed only when the flies' eggs were deposited on the meat itself. Obviously the meat itself did not turn into maggots. Redi's work was not widely noted, however, and it was not until much later that another series of experiments was made.

**Louis Joblot (1645-1723).** After Leeuwenhoek's discovery it was thought by many who believed in the spontaneous generation of life that animal or vegetable matter contained a "vital or vegetative force," capable of converting such matter into new and different forms of life. Leeuwenhoek's "animalcules" were hailed by many as proof of this. In 1710, Louis Joblot observed that hay, when infused in water and allowed to stand for some days, gave rise to countless animalcules or infusoria (bacteria and protozoa). The hay was thought to change into "animalcules." Anyone can observe the development of the "animalcules" for himself. In those times, however, most people regarded this as conclusive evidence of spontaneous generation. Joblot, however, boiled hay infusion and divided it into two portions, placing one in a carefully baked (*sterilized*) and closed vessel, the other in an open vessel. The infusion in the open vessel teemed with living creatures in a few days. In the closed vessel no life appeared as long as it remained closed, thus showing that the infusion alone, once freed of life by heat was incapable of generating life anew spontaneously.

**John Needham (1713-1781).** Similar experiments, carried out by an English scientist, John Needham (1749), gave conflicting results. Life developed in

\* The student will note three main phases of the question of spontaneous generation: (a) That concerning the formation of complete and highly developed animals like bees, mice, frogs and the like from putrid matter, mud, cheese, dirty linen, etc.; (b) that which concerns the cause of putrefaction and fermentation; and (c) that concerning the origin of life through chemical evolution early in the history of life on this planet.



Needham's heated, closed vessels as well as in the open, unheated ones. He, therefore, believed in spontaneous generation. We shall see later that this result was due to insufficient heating, which failed to kill heat-resistant forms of bacteria called *spores*. But nothing was known about spores at that time.

**Lazzaro Spallanzani (1729–1799).** Spallanzani, an Italian naturalist, published the results of a whole series of the same type of experiments, which disagreed with those of Needham. He showed that if heating was prolonged sufficiently, and the vessels kept closed to exclude dust and air, no animalcules developed in hay infusions, or in any other kinds of organic matter like urine and beef broth. Needham, in reply, said the prolonged heating destroyed the “vegetative force” of the organic matter which, he said, was necessary for the spontaneous generation of life. Spallanzani answered Needham's objections by showing that the heated infusions in the closed flasks could still develop animalcules *when exposed to air* (i.e., when microorganisms were introduced with dust).

In 1775 Lavoisier discovered *oxygen* and the relation between air and life. This renewed the controversy about spontaneous generation, the objection to Spallanzani's results being raised that it was the exclusion of air (oxygen) from the flasks which prevented the development of life.

**Schulze and Schwann.** New experiments were performed, in which air was admitted to the previously heated infusions of meat or hay, but only after passing through sulfuric acid or potassium hydroxide solutions (Schulze, 1836) or through very hot glass tubes (Schwann, 1836), the idea being that *the air itself introduced the germs of life into the infusions*. When the infusions exposed to air so treated failed to develop any life, it was claimed by others that this was not due to a destruction of any germs of life in the air by the sulfuric acid or hot glass, but to the fact that the “life-giving” power of the air had been destroyed by these methods, thus preventing spontaneous generation. Schröder and von Dusch (1854–61) overcame this objection by performing similar experiments in which the air was merely filtered through cotton wool. This method prevented the appearance of animalcules in the heated broth or infusions until the vessels were opened. It was therefore apparent that the method of treatment of the air had nothing to do with the development of animalcules, *and that these did not develop spontaneously* but that there were particles of living matter, floating on dust in the air, which not only were killed by heat, acids and alkalis but which could be caught and withheld by the cotton wool alone. The presence of the microorganisms in the cotton wool was later proven by Pasteur. The experiments of Schröder and von Dusch were the origin of our present-day use of cotton plugs for bacteriological culture tubes and flasks.

In spite of these demonstrations long and bitter controversies still raged. Schröder and von Dusch were themselves not convinced by their own experiments and admitted the possibility that spontaneous generation might occur under natural conditions.

**Louis Pasteur (1822–1895).** Pasteur, the most famous, and considered by many the greatest, French scientist of all time, was born in Dôle, December 27, 1822. Son of a moderately prosperous tanner who had fought for, and been decorated on the battlefield by, Napoleon, Pasteur had a great admiration for his father's soldierly accomplishments. He later was moved to many



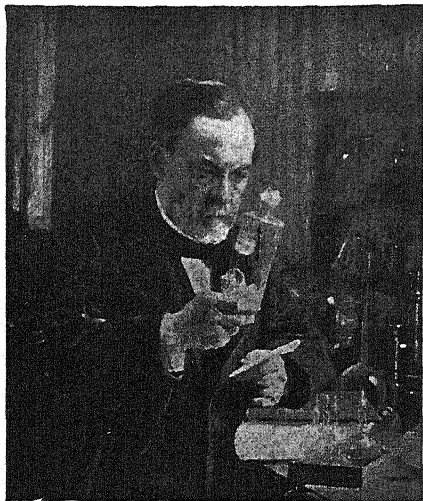


Fig. 2-5. Pasteur in his laboratory. Dr. Pasteur is examining a specimen of spinal cord from a rabid animal. The material at the bottom of the jar is sodium hydroxide (drying agent).

of his best scientific achievements by his patriotic zeal. In his boyhood he was an indifferent student but later became an enthusiastic scholar, devoting his energies to a study of chemistry. He discovered the relationship of the stereo-isomeric forms of tartrate crystals and revealed a whole new series of possibilities in physical chemistry.

Pasteur, however, was not one to gloat over such successes and rest on his laurels. He sought for other fields of investigation. His choice was guided largely by patriotic motives. An Englishman had said to him: "People are astonished in France that the sale of French wines should not have become more extended here [in England] since the Commercial Treaties. The reason is simple enough. At first we eagerly welcomed French wines, but we soon had the sad experience that there was too much loss occasioned by the diseases [souring] to which they are subject."\* Germany was, at that time, making a much better beer than France, and Pasteur undertook to make France a successful rival in that respect. In order to do so he made a long study of beer manufacture and of the cause of souring and spoilage ("diseases") of beer and wines. As a result of these studies he arrived at far-reaching conclusions. "Might not the diseases of wines," he said at the Académie des Sciences in January, 1864, "be caused by organized ferments; microscopic vegetations, of which the germs would develop when certain circumstances of temperature, of atmospheric variations, of *exposure to air*, would favour their evolution or their introduction into wines? . . . I have indeed reached this result, that the alterations ("diseases") of wines are coexistent with the presence and multiplication of microscopic vegetations." Pasteur had found that acid wines, "ropy" wines, bitter wines, sour beer and so on, were due to the growth in them of undesirable contaminating organisms which produced these so-called "diseases."\*

The solution of the problem, as later proven by Pasteur (Fig. 2-5), lay in

\* From "The Life of Louis Pasteur," by René Vallery-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.

preventing the growth of foreign organisms, "wild" yeasts, bacteria, etc., which caused the undesirable conditions. After considerable experimentation along these lines he discovered that the wine did not spoil in transit, if it were held for some minutes at a temperature between 50° C. and 60° C. He said, "I have . . . ascertained that wine was never altered by that preliminary operation (heating), and as nothing prevents it afterwards from undergoing . . . improvement with age—it is evident that this process (heating) offers every advantage." His experiments were so successful that a practical test of the efficacy of his methods was made. He wrote to a friend, ". . . experiments on the heating of wines will be made by the Minister of the Navy. Great quantities of heated and nonheated wine are to be sent to Gabon so as to test the process; at present our colonial crews have to drink mere vinegar."\*

Pasteur laid down three great principles:

1. Every alteration, either of beer or of wine, depends on the development in it of microorganisms which are ferments of "diseases" of the beer or wine.
2. These "germs or ferments" are brought by the air, by the ingredients, or by the apparatus used in breweries.
3. Whenever beer or wine contains no *living* microorganisms it remains unchanged.

In the same way that wines could be preserved from various causes of alteration by heating, bottled beer could escape the development of disease ferments by being brought to a temperature of 50° C. to 55° C. "The application of this process gave rise to the new word, 'pasteurized' beer, a neologism which soon became current in technical language." Today, pasteurization of milk (heating at 62° C. for thirty minutes) is commonplace. The heating kills undesirable microorganisms.

"Pasteur foresaw the distant consequences of these studies, and wrote in

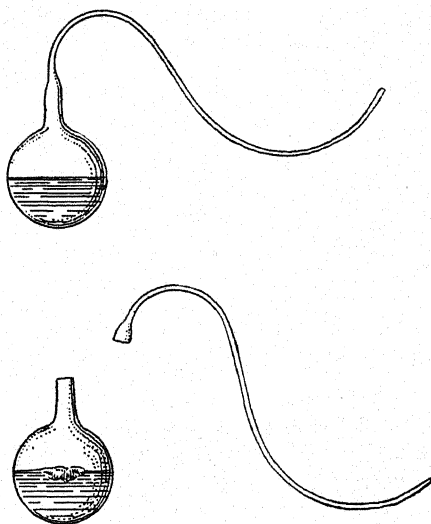


Fig. 2-6. Two of Pasteur's flasks, showing open neck with curves to catch dust. One flask has been opened and a growth of mold has developed. (Duclaux, "Pasteur, The History of a Mind.")

\* From "The Life of Louis Pasteur," by René Vallery-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.



Fig. 2-7. First illustration of amputation, from Von Gersdorff (1517). Bandages constrict, at most, superficial veins. Two arteries are spurting uncontrolled. Man in background is wearing Gersdorff's pig's bladder dressing over his forearm stump. Bacteria had a field day under such conditions!

his book on beer—"When we see beer and wine subjected to deep alterations because they have given refuge to microorganisms invisibly introduced and now swarming within them, it is impossible not to be pursued by the thought that similar facts may, *must*, take place in animals and in man."

It was obvious from Pasteur's studies that each special kind of fermentation or disease of beer or wine was the result of the growth and activity in it of a *special, distinct* form of yeast or other microorganism depending on the type of fermentation or disease under investigation. This furthered an idea, already old, of the *specificity* of biological action, and supported the view that animal and human diseases also, like different sorts of putrefaction and fermentation, were each caused by a single, specific type of microorganism.

After Pasteur's views with regard to the nature of fermentation had been made public, he became involved in the bitter quarrel over the apparently mysterious appearance of the "germs" in fermentable or putrescible liquids like wine, beer, urine, broth, etc., hitherto regarded by many (Needham and others) as resulting from spontaneous generation. Without going into detail which would occupy too great a space, we may cite a series of experiments which Pasteur carried out to answer the various objections and fallacies of previous workers, and to show that the "animalcules" in spoiled beer, wine, etc., were merely descendants of microorganisms which had gained access to the fluids from dust in the air and which, by their growth and metabolism, caused fermentation and putrefaction. First he redemonstrated that living creatures float in the air attached to particles of dust. Then he showed, as Schulze and Schwann had done, that when they could be excluded from various things like sterilized broth and urine, these substances did not ferment or putrefy. By using flasks with long, open necks having several vertical bends in them, he showed that, although unheated and untreated and unfiltered air communicated freely with the interior, the dust was caught by gravity in the bends of the neck and no life appeared in the infusions (Fig. 2-6). Not until

the flask was tilted so that the fluid came into contact with this dust and was allowed to run back into the flask, or until the neck of the flask was broken off close to the body, did growth occur in the fluids. Some of Pasteur's flasks which were sterile in 1864 have been preserved and are still sterile (if they have not been destroyed by wars) after over 100 years!

**Lord Lister (1827–1912).** One of the many important applications of the work of Pasteur was made in 1867 by the English surgeon Lister. He realized that wounds become infected, during surgical operations, by bacteria floating on particles of dust in the air, or clinging to instruments or to the hands of the surgeon (Fig. 2–7). Obviously, in order to prevent such wound infections it was only necessary to render all surgical appliances sterile and, by antiseptic dressings and careful technique, completely to prevent the entrance of bacteria into surgical wounds. Lister's original method of preventing infection during surgical operations was to work in a field and atmosphere continuously wet with a fine mist of carbolic acid solution emanating from a nearby apparatus (Fig. 2–8). This must have made the surgeon's work difficult but the results obtained were the foundation of our modern, aseptic surgery (Fig. 2–9). The student may judge for himself whether Lister's contribution was of importance to human life and medicine.

#### THE BEGINNINGS OF PRECISE BACTERIOLOGY

One reason, perhaps, why more species of bacteria were not discovered by Pasteur and others prior to about 1870 was that microbiological methods were very crude. In nature, microorganisms seldom occur alone, or "pure," but in mixtures of many species together. It was very difficult, at that time, to separate, in a pure and uncontaminated state, any given sort of microorgan-

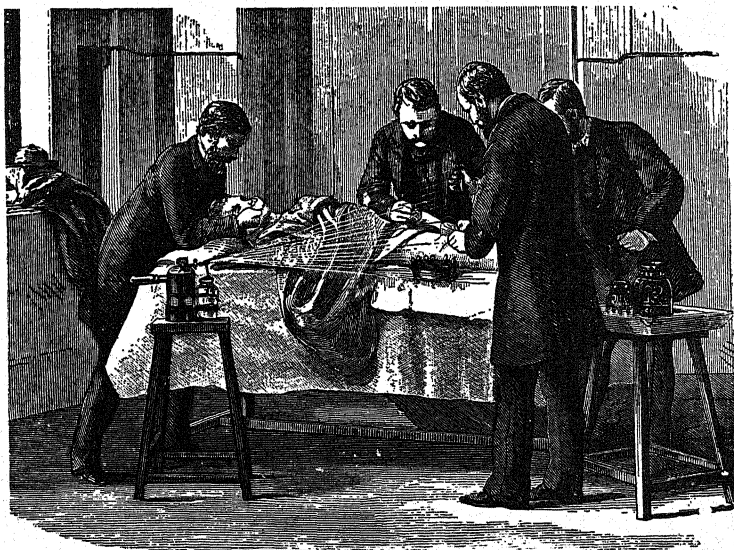


Fig. 2–8. Lister operating with carbolic spray. Representing the general arrangement of surgeon, assistants, towels, spray, etc., in an operation performed with (supposed) complete aseptic (antiseptic) precautions (1882). Note the carbolic spray playing over the field of operation. (W. Watson Cheyne.)

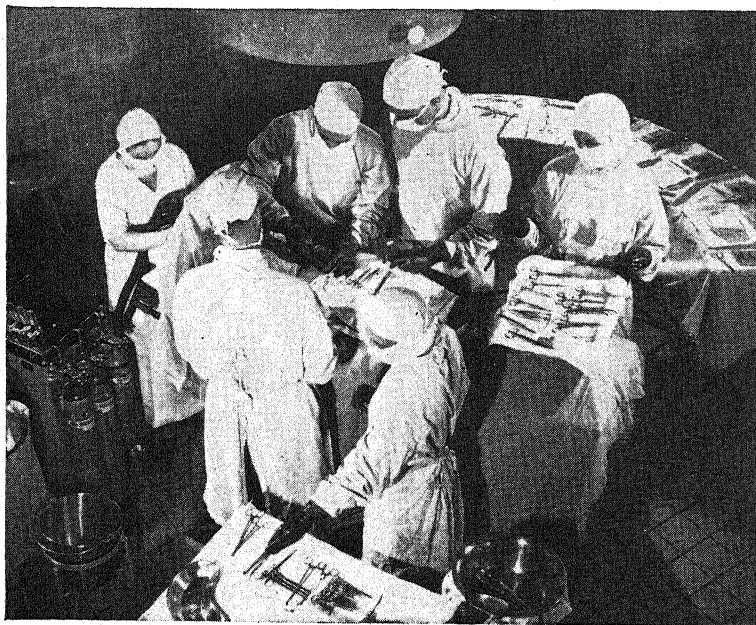


Fig. 2-9. Modern surgery. Note the sterile masks, caps, gowns, rubber gloves, and sheets. The instruments have all been sterilized. (St. John's Hospital, Brooklyn. Courtesy Ewing Galloway.)

ism from a mixture of microorganisms. The preparation of such "pure cultures" is absolutely essential to the exact study of microorganisms just as pure substances are necessary to the analytical chemist. For the development of pure culture technique, and for many of our modern bacteriological methods we are indebted to that precise German scientist, Robert Koch.

**Robert Koch (1843-1910).** While Pasteur, and others were engaged in their polemics and experiments on fermentation and spontaneous generation, Koch was practicing medicine and was Provincial Health Officer in Wollstein. He had occasion, in an official capacity, to investigate anthrax (a disease of animals and man caused by *Bacillus anthracis*, a species of cylindrically-shaped bacteria). He decided to study the disease in his laboratory during his spare time.

At first Koch and other scientists examined all of their microorganisms in the living state, usually in drops of fluid mounted on a bit of glass. They thus became familiar with bacterial motility when present, and they observed refractile granules inside various bacteria. But the constant motion of some of the bacteria (either that purposeless oscillation, due to molecular impact and known as brownian movement, or real, progressive motion due to the action of flagella) as well as their transparency, made accurate and prolonged study most difficult. Koch realized that it would be much better for his drawings, and especially for his photographs (both of which, by the way, were excellent; Fig. 2-10) if the bacteria could be made to remain still. He tried spreading out his drops of anthrax-infected fluid in thin films and allowing them to dry, and met with immediate success. Not only were the anthrax bacilli motion-

less, but they apparently had not shriveled or changed in any visible way. However, the bacteria were transparent and colorless. It was very difficult to observe the fine details of their structure, and equally difficult to photograph them. He obtained ideas from other workers, a procedure commended to all investigators.

#### DEVELOPMENT OF STAINING METHODS

**Weigert and Ehrlich.** Weigert, a German scientist contemporary with Koch, had observed the use, by Cohn and others, of various dyes to make clear the details of cell structures in histological preparations (histology = microscopic anatomy). This procedure had been in use for some time, the natural dyes *carmine* and *hematoxylin* being widely used. Ehrlich, a renowned chemist, had recently improved methods, discovered by William Perkin, a brilliant British chemist, of preparing very fine dyes from coal-tar distillates. These were the first "coal-tar" or aniline dyes. Weigert, the bacteriologist, tried the methods of the histologists with the dyes invented by the chemist. His first success was in 1875, when he found that the dye *methyl violet* could be used to reveal bacteria in histological preparations. This method of making bacteria easy to find and study, where before they had been colorless and transparent and therefore almost invisible, was adopted by Koch and soon came into wide use. Modified, it is one of our best methods today.

**Fluorescence Microscopy.** A very interesting modern variation on this theme is the use of fluorescent compounds to stain certain specific kinds of

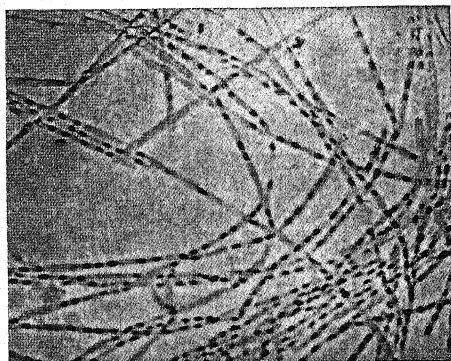
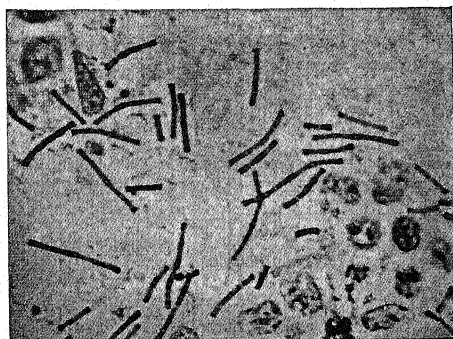


Fig. 2-10. Koch's photograph of anthrax bacilli.

The upper picture shows the bacilli containing endospores (dormant, heat-resistant bodies) and strung together in long chains sometimes called streptobacilli. The lower picture shows the bacilli as individual cells and in short chains of 2 to 6 cells. Many present-day microphotographs are no better than these taken about three quarters of a century ago.





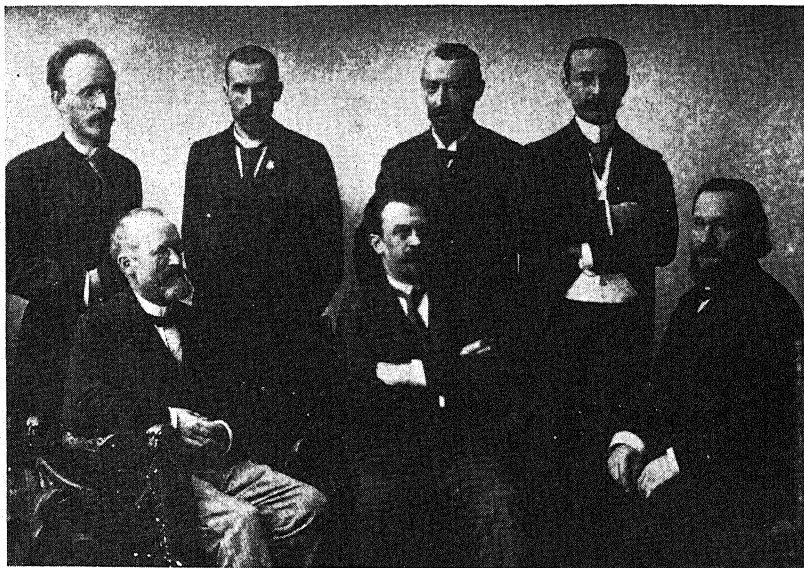


Fig. 2-11. One of the groups of famous scientists who studied microbiology under Koch. Standing, left to right: Alphonse Laveran (1845-1922), discoverer of the malarial parasite; Emile Roux (1853-1933), codiscoverer of diphtheria toxin; Edmund Étienne Nocard (1850-1903), French veterinarian and mycologist; George H. F. Nuttall (1862-1937), British microbiologist. Sitting, left to right: Robert A. Koch (1843-1910), discoverer of the tubercle bacillus and pioneer microbiologist; Karl Joseph Eberth (?) (1835-1926), discoverer of the typhoid bacillus; Elie Metchnikoff (1845-1916), Russian zoologist and discoverer of phagocytes and phagocytosis. (Courtesy of Wiley A. Penn, Director of Laboratories, Department of Health, Savannah, Georgia.)

bacteria which are particularly difficult to see under ordinary conditions. When the fluorescent organisms are illuminated with ultraviolet light and viewed through a microscope they are as easily found as bright stars in the midnight sky! This is called *fluorescence microscopy*.

**Development of Gram's Stain.** In 1884, a Danish scholar, Gram, found that if anthrax bacilli were stained with Ehrlich's methyl-violet solution, and then treated with iodine, the dye was "fast" and that even alcohol could not remove it. In attempting to apply the method to certain other bacteria, however, he found that the dye was not fast and that as soon as alcohol was applied the color was removed and the bacteria were as colorless and transparent as ever. They could even be dyed an entirely different color (counterstained) with some other dye. The bacteria retaining the purple stain were called gram-positive, while the others were called gram-negative. The method is now used, generally with some slight modification, by every bacteriologist as one of the first steps in the process of identifying an unknown bacterium.

Koch, using the methods of Ehrlich, Weigert, Cohn, Gram, Petri (see Chap. 13) and others, became one of the foremost bacteriologists of his day. His discoveries attracted scholars from all over the world (Fig. 2-11). In another chapter there is outlined the epoch-making development of solid media and the covered plate for the pure-culture cultivation of bacteria, which are methods used daily in every bacteriological laboratory in the world. The

methods and principles which originated in the laboratories of Koch, Pasteur, Lister and others have made possible the conquest of disease and the building up of great industries. Greatest of all, they have left us a foundation of fact and inspiration upon which to build greater achievement and this, possibly, is the richest legacy which any man can leave to posterity.

**Other Developments.** Leeuwenhoek, Pasteur, Koch and their contemporaries laid foundation stones of basic importance in the science of Microbiology and their names are writ large in the Halls of Fame. However, it must not be supposed that they were the last great microbiologists. They were among the *first*. Later workers, equally ingenious, learned and courageous, have carried on the work along an ever-increasing number of lines of investigation. Although the five years following Koch's demonstration of the value of solid media in plates for isolating bacteria witnessed the discovery of the organisms causing diphtheria, cholera, erysipelas, lockjaw, pneumonia and a number of other diseases, discovery of a new species was, by that time, only a matter of patient "plating out" of suspected materials and was only one, rather limited, phase of the subject.

One of the greatest discoveries in medical history was made when, in 1890, von Behring, Kitasato and Fraenkel found that guinea pigs could be immunized by injections against tetanus and diphtheria toxins and that their blood then contained antitoxin which would protect other animals. The extension of this principle to other diseases was an obvious possibility and soon bore fruit.

In the realm of soil microbiology, equally important advances were going on simultaneously. Beijerinck, in 1888, had already isolated the bacteria which, in the nodules of leguminous plants, aid them in the direct utilization of atmospheric nitrogen, a physiological property essential to the existence of most other forms of life. Winogradsky, Beijerinck, Omeliansky and others during the decade following 1890 worked out the processes by which ammonia, formed in the soil by various biological processes, is oxidized by successive stages, first into nitrites by organisms of the genera *Nitrosomonas*, *Nitrosococcus*, etc., and then into nitrates by bacteria of the genera *Nitrobacter* and *Nitrocystis*. As nitrate, nitrogen is readily available to most plants of agricultural value and without it the human race might have had a very different history. The equally important transformations in the soil of sulfur, carbon and other elements were also studied by these workers.

In 1893 to 1894 Winogradsky demonstrated that atmospheric nitrogen is fixed in the soil by the anaerobic bacterium *Clostridium pasteurianum*. Aerobic organisms having a like property, *Azotobacter chroococcum* and *A. agile*, were discovered by Beijerinck in 1901. These organisms are of absolutely fundamental importance to the history of the human race since, without fixation of atmospheric nitrogen in the soil, there would be no human race at all. This rather deflationary fact will be explained later.

In 1892 Iwanowski discovered the existence of a disease-producing agent (virus of tobacco-mosaic) which was invisible and not cultivable on inanimate media and which traversed filters capable of holding back the smallest bacteria. Many other viruses have since been discovered and great strides have been made in our knowledge of that class of organisms. For example, Walter Reed in 1900 to 1902 demonstrated not only the mode of transmission but the



viral nature of the cause of yellow fever, the first-known viral disease of man. Later, about 1933, workers of the International Health Division of The Rockefeller Foundation developed a fully effective vaccine against yellow fever and to date it has been given to millions of persons.

A whole series of interesting and valuable discoveries has resulted more or less directly from the studies of Koch, Ehrlich, Weigert and the others with the staining of bacteria. From continued investigation of the effects of dyes upon bacteria there has arisen, from the work of Domagk (Nobel prize winner) 1932, knowledge of such dye-like compounds as sulfanilamide and sulfadiazine, the use of which in treating bacterial infections is a matter of common knowledge. In 1929 Sir Alexander Fleming (Nobel prize winner) made the momentous discovery of penicillin, and Waksman (Nobel prize winner) et al., a little later, discovered streptomycin, thus opening up the huge field of antibiotics, the importance of which is still growing. In 1949 Enders, Robbins and Weller (Nobel prize winners) showed how to cultivate poliomyelitis virus in monkey tissues in flasks. By 1955 the polio vaccine developed on this basis by Salk (awarded a Presidential citation and Congressional Medal) was known and used all over the world. Each began as a young student, making notes in a classroom, listening to teachers who had trod the paths before him; trying, failing at times, persevering, succeeding.

So Science grows. The microbiological history of tomorrow is being written in the laboratory notebooks of students today. And who knows which notes will prove to be the basis of the greatest discovery?

#### REFERENCES

- Allison, V. D.: Sir Alexander Fleming. Obituary Notice. *J. of Gen. Microb.*, 1955, 13:1.  
 Aron, H. C. S.: Paul Ehrlich: His contributions to medicine. *J.A.M.A.*, 1954, 154:969.  
 Barrell, Schuchert, et al.: *The Evolution of the Earth*. Yale University Press, New Haven, 1922.  
 Beutner, R.: *Life's Beginnings on the Earth*. Williams & Wilkins Co., Baltimore, 1938.  
 Bulloch, Wm.: *The History of Bacteriology*. Oxford University Press, London, 1938.  
 Calvin, M.: Chemical Evolution and the Origin of Life. *Am. Sci.*, 1956, 44:248.  
 Chapman, V. J.: *Seaweeds and Their Uses*. Pitman, New York, 1952.  
 Cohen, B.: On Leeuwenhoek's method of seeing bacteria. *J. Bact.*, 1937, 34:343.  
 Cohen, B.: *The Leeuwenhoek Letters*. Society of American Bacteriologists. Williams & Wilkins Co., Baltimore, 1937.  
 Cohn, F.: *Bacteria: The Smallest of Living Things*. (Trans. C. S. Dolley.) Johns Hopkins Press, Baltimore, 1939.  
 Dobell, C.: *Antony van Leeuwenhoek and his Little Animals*. Harcourt, Brace and Co., New York, 1932.  
 Dodson, E. O.: *A Textbook of Evolution*. W. B. Saunders Co., Philadelphia, 1952.  
 Dublin, L. I.: Robert Koch, A Centenary, 1843-1943. *The American Scholar*, 1943-44, 13:95.  
 Dubos, R. J.: *Louis Pasteur, Free Lance of Science*. Little, Brown & Co., Boston, Mass., 1950.  
 Ford, W. W.: *Bacteriology*. Paul B. Hoeber, Inc., New York, 1939.  
 Fred, E. B.: Antonj van Leeuwenhoek. *J. Bact.*, 1933, 25:1.  
 Goodlee, R. J.: *Lord Lister*. The MacMillan Co., New York, 1918.  
 Haggard, H. W.: *Devils, Drugs and Doctors*. Garden City Publishing Co., Garden City, New York, 1929.  
 Haldane, J. B. S.: *The Origin of Life*. In "The Inequality of Man." Harper and Bros., New York, 1928.  
 Horowitz, N. H.: On the evolution of biochemical syntheses. *Proc. Nat. Acad. Sci.*, 1945, 31:153.

- Kudo, R. R.: Protozoology. 4th ed. Charles C Thomas, Springfield, Ill., 1954.
- Lamanna, C., and Mallette, M. F.: Basic Bacteriology. Williams & Wilkins Co., Baltimore 1953.
- Locy: Biology and Its Makers. 3rd ed. Henry Holt and Co., New York, 1930.
- Muller, H. J.: Life. Science, 1955, 121:1.
- Oparin, A. J.: The Origin of Life. (Trans. Morgulis). The MacMillan Co., New York, 1938.
- Read, J.: Sir William Perkin. Sci. Am., 1957, 196:110.
- Schröder, H., and van Dusch, T.: Ann. d. Chem. u. Pharm., 1854, 89:232.
- Vallery-Radot, R.: The Life of Pasteur. (Trans. Devonshire.) Doubleday-Doran and Company, Inc., New York, 1926.
- van Niel, C. B.: The "Delft school" and the rise of general microbiology. Bact. Rev., 1949, 13:161.
- van Niel, C. B.: In memoriam Prof. Dr. Ir A. J. Kluiver. Antonie v. Leeuwenhoek J. Serol. and Micr., 1956, 22:209.
- Waksman, S. A.: Contribution of "A Simple Bacteriologist" to humanity. (Obit. Sir Alexander Fleming.) Science, 1955, 121:580.
- Waksman, S. A.: Sergi Nikolaevitch Winogradsky: 1856-1953. (Obit.) Science, 1953, 118:36.
- Winslow, C-E. A.: Some leaders and landmarks in the history of microbiology. Bact. Rev., 1950, 14:99.

## The Microscopic World

### 3. YEASTS AND MOLDS

#### GENERAL CHARACTERISTICS

ALTHOUGH NEITHER yeasts nor molds are included in the class Schizomycetes, they belong in the same major division of the vegetable kingdom, namely, the Mycophyta or fungi. Many yeasts and the structural features of molds are microscopic in size and the microscope is used in studying them. They make very good study material for microbiology because of the relatively large size and evident structural details of their cells.

The place of yeasts and molds in the organic system is fairly well defined. They are distinguished from *algae* by their lack of chlorophyll and growth in the dark. They are readily differentiated from *protozoa* by their characteristic morphology and by the motility of most protozoa; also by their ability to grow readily on artificial media which protozoa rarely do. Their relatively large size and obvious nuclear structures readily distinguish them from bacteria. There is no chance of confusing them with PPLO, *viruses* or *rickettsia* because of differences in size and structure and failure of the last two to grow on an artificial media (see Table 1).

Many common species of yeasts and molds are of great importance in industry. Some cause fermentations which yield valuable substances like ethyl and isopropyl alcohols, acetone, etc. Others cause damage through decay, "mildew," etc. Molds are exceedingly active in the decay of wood and other organic matter. Their destructive action on wood in damp soil is a cause of considerable economic loss to telephone and electric power interests as well as to farmers with wooden fence posts. These, therefore, are concerned with experiments with antifungal preservatives of wood (Fig. 3-1). Many molds and yeasts are causes of diseases of plants, animals and man. Some molds, such as *Penicillium*, have assumed enormous importance as the sources of antibiotics. Molds and yeasts are often encountered by the bacteriologist as contaminants in laboratory cultures\* because the fungi are ubiquitous and their spores or conidia are constantly present in dust, soil, air, etc.

\* A *culture* of microorganisms consists of a flask, tube or other vessel containing a nutrient material such as meat-broth, milk or other food substance (solid or liquid) in (or on) which microorganisms are growing. The nutrient is spoken of as a *medium* or *culture medium* (pl. = media). A *pure culture* is one in which only one kind of microorganism is growing. A *contaminated culture* is one to which an unwanted, extraneous microorganism has accidentally gained entrance.

Yeasts and molds may be cultivated on much the same sort of materials as are used for bacteria and they are stained and manipulated in much the same way. Certain media are especially recommended for the cultivation of these organisms, a good illustration of the general type being that of Sabouraud.\* Most of the media of choice for molds and yeasts contain considerable amounts of carbohydrate. The preferable reaction of this type of medium is slightly acid (pH about 5.5). Slight acidity seems to favor the growth of many molds and yeasts and inhibits the growth of some kinds of contaminating bacteria. In general, the yeasts and molds have nutrient requirements similar to those of saprophytic† bacteria.

Structurally and in methods of reproduction yeasts and molds are more complex than bacteria and may, therefore, be regarded as more highly evolved plants.

**Classification of Fungi.** Differentiation between yeasts and molds is sometimes difficult because the transition in form and in manner of reproduction, from one group to another, is so subtle that it is difficult to draw sharp lines of demarcation.

For present purposes it is sufficient to indicate the main divisions of the group of fungi, leaving finer systematization for more advanced students of



Fig. 3-1. Blocks of wood taken from telephone poles preserved by different methods. The wood remains on the damp soil under conditions simulating actual exposure of poles to the action of soil microorganisms; especially fungi. Such tests are part of the search for better wood preservatives. (Photo courtesy of Bell Telephone Laboratories, New York.)

\* Sabouraud's agar:

Water.....	1000 ml
Peptone.....	10 gm
Glucose (or Maltose).....	40 gm
Agar.....	20 gm

Dissolve all ingredients in water. Sterilize by autoclaving. The pH should be about 5.0-5.6.

† Causing decomposition of dead organic matter. Generally harmless.

the subject. The following outline, while incomplete, is a satisfactory working basis for the present discussion. (See Table 2.)

**Table 2.** *Classification of Fungi*

Thallophyta (no stems, roots, flowers, leaves)				
Algae (chlorophyll)		Fungi (no chlorophyll)		
Schizomycetes (bacteria)		Eumycetes yeasts, molds, mushrooms, puff- balls, etc.		
Phycomycetes Not sac-formers.	Ascomycetes Sac-formers.	Fungi imperfecti Properties of both yeasts and molds.		Basidiomycetes Not sac-formers.
Mycelia not sep- tate.	Yeast forms Mainly unicell- ular or bud- ded.	Filamentous forms Mycelia septate.	Fungi having no observed sexual cycle are found in this group.	Mycelia septate.
Asexual spores held in sporangia.	Asexual spores blastopores.	Asexual spores conidia.	Asexual spores conidia of many types.	
Sexual spores free (zygo- spores or oospores).	Sexual spores in asci.	Sexual spores in asci.	No sexual phe- nomena known.	Sexual spores on basidia.
Familiar exam- ples:				
Aquatic forms cause diseases on fish and plants. Example: <i>Sapro- legnia</i> .	<i>Sacchuromyces</i> <i>Schizosaccharo- myces</i> (Above are typi- cal yeasts).	<i>Aspergillus</i> <i>glaucus</i> (green mold, common on bread). <i>Penicillium itali- cum</i> (blue mold, com- mon on de- caying citrus fruit).	Many fungi causing plant diseases belong in this group. Many fungi caus- ing diseases of man and ani- mals also are placed here pending more complete stud- ies.	Mushrooms. Bracket or tree fungi. Rusts (e.g., wheat rusts). Smuts (e.g., corn smuts).
Common terres- trial forms are <i>Mucormucedo</i> ("manure mold," white) and <i>Rhizopus</i> <i>nigricans</i> ("bread mold," black).				

### THE YEASTS

Ordinarily we think of yeasts as unicellular, microscopic plants with ovoid or elliptical cells, each cell living as a separate, complete individual. We often differentiate yeasts from molds by the fact that molds typically form greatly

elongated structures growing in branched filaments or *hyphae* and forming tangled masses of hyphae called *mycelia*. A difficulty in such classification arises from the fact that some yeasts form more or less definite mycelia under certain conditions of growth and nutrition; i.e., they are *dimorphic*.

**Dimorphism.** The ability of some yeast-like fungi to grow in either (1) the yeast (Y) phase or (2) the filamentous (F) phase is spoken of as *dimorphism*. Various factors may induce this phenomenon. For example, low temperature and ageing generally favor the F phase, while certain nutrients (blood, glucose, compounds with reduced sulfur, like -SH groups, etc.) or absence of air (anaerobiosis) often favor the Y phase. There are various substances (fusel oil, cobalt ions etc.) which induce the Y  $\rightarrow$  F variation. Camphor exhibits this effect so markedly that its action has been called the CF reaction.

The Y  $\rightleftharpoons$  F variation in yeast-like fungi may be related to the smooth (S)  $\rightleftharpoons$  rough (R) variation in bacteria; the Y and F phases of yeasts corresponding to the S and R phases of bacteria, respectively.\*

**Structure of Yeast Cells.** Yeast cells are, on the average, much larger than bacterial cells. Some oval yeast cells have a volume hundreds of times that of *Micrococcus*† cells and a long diameter up to 20 $\mu$  or more. There is a well-differentiated nucleus and a thick cell wall, composed of cellulose-like substance. Yeast cells may, by appropriate techniques, be sliced into *exceedingly thin* sections. These are stained differentially and then examined in enlarged photos of electron microscope pictures (electronographs) at magnifications up to 100,000 diameters. It is possible in such preparations to see that the yeast cell wall consists of at least two, and probably three or more, layers. Within the cytoplasm are numerous vacuoles containing food or waste substances and there are granules of various kinds, some evidently related to the formation and storage of glycogen, others composed of volutin (Figs. 3-2, 3-3). Yeasts sometimes contain large quantities of fat, of which commercial use may be made.

On the other hand, yeasts resemble most bacteria in being unicellular, non-motile, devoid of chlorophyll, and plant-like in requiring food material which is wholly soluble and which can pass through the cell wall only by diffusion.

**Multiplication of Yeasts.** Yeasts may multiply by one or more of four methods.

1. **BUDDING.** First, they multiply by a method called *budding* in which large, mature cells divide, each giving rise to one or more daughter cells which are at first much smaller and which may cling to the parent cell (Fig. 3-3), often even after the daughter cell has divided. Clumps and chains of cells sometimes called rudimentary filaments or pseudomycelia, are thus formed.

2. **FISSION.** Some species of yeast, in the genus *Schizosaccharomyces*, divide by *equal (binary) fission*, much as do the bacteria.

3. **ASCOSPORES.** *Ascospores* (spores within a sac or *ascus*) are formed within a single cell when the nucleus undergoes 1, 2 or 3 divisions without participation of the cell wall, forming 2, 4 or 8 ascospores in the sac. There is no apparent involvement of sexes in this process. Because yeasts form spores en-

\* If you are familiar with genetics it is of interest to note there is a suggestion of a parallelism between Y  $\rightleftharpoons$  F in molds and yeasts, R  $\rightleftharpoons$  S in bacteria and the haploid  $\rightleftharpoons$  diploid state.

† Common, spherical bacteria about 1  $\mu$  in diameter.

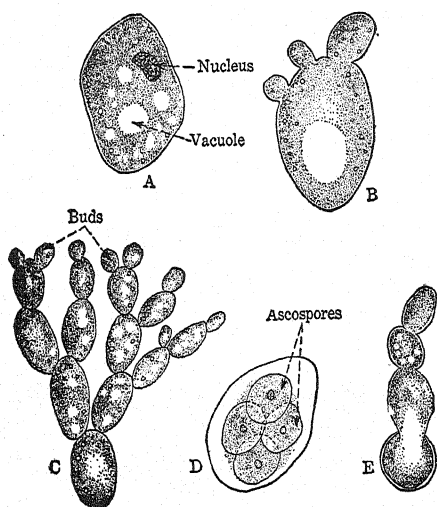


Fig. 3-2. Yeast (*Saccharomyces cerevisiae*). A, single cell highly magnified; B, cell in process of budding; C, chain of cells formed as result of rapid budding and growth; D, formation of ascospores; E, germination of ascospore and the development of new plants by budding. (Redrawn from Curtis. Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," John Wiley & Sons, Inc., publishers.)

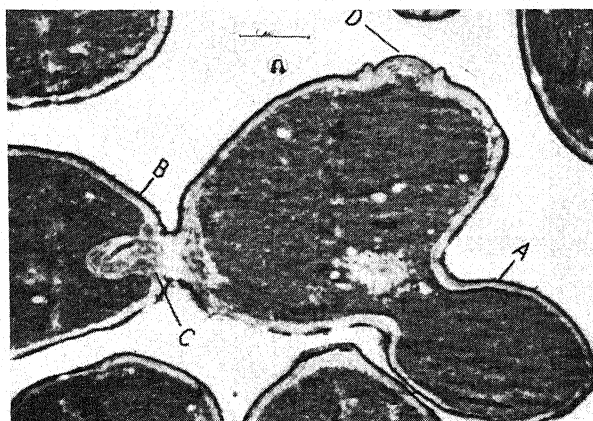


Fig. 3-3. Electronograph of a longitudinal section through a budding yeast cell (*Saccharomyces cerevisiae*). A is a bud with its cytoplasm continuous with that of the mother cell. B is a mature bud with the developing cross wall between mother and daughter cell. C is the extension of cell wall material into the cytoplasm; a phenomenon which appears to be characteristic of the later stages of the budding process. D is a bud scar, the surface of which is always convex. (From the collection of the Society of American Bacteriologists, courtesy of Hilda D. Agar and H. C. Douglas in *J. Bact.*, 1955, vol. 70.)

closed in an ascus (Fig. 3-4), they are classed in the group of Ascomycetes or sac-forming fungi.

The ascospores are, in some respects, analogous to bacterial spores, being resistant to climatic heat, drought, and other unfavorable environmental conditions. They are not so thermoresistant as bacterial spores, being killed by 60° C in a short time. Bacterial spores resist boiling and even much higher temperatures for hours. Since ascospores of yeasts are generally produced in groups of two or more per cell, they represent a process of multiplication as

well as preservation, thus differing from bacterial spores, of which only one is produced by each individual cell.

4. **SEXUAL PROCESSES.** The sacs or asci of yeasts of many species often result from readily discernible *sexual* processes in which two cells send out projections which meet and form a copulation canal; the two cells form a zygospore.\* The nucleus of this cell divides within the sac to form a number of ascospores (Fig. 3-5).

**CHLAMYDOSPORES.** Another type of asexual spore is called chlamydospore (*Chlamydo* is from a Greek root meaning a protective covering). Many yeasts and molds (and probably bacteria) are capable of forming these bodies (Fig. 4-3). Active growth of the cell ceases, food is stored, the cell acquires a thick protective wall, dehydration takes place, and the resulting dormant cell tides the plant over unfavorable conditions. No increase in numbers of nuclei occurs as in ascospore formation and the chlamydospore is therefore *not reproductive* in function. Such bodies are not as heat-resistant as bacterial spores.

**Habitat of Yeasts.** Yeasts and yeast-like fungi are widely distributed in nature. They commonly occur on grapes and other fruits, vegetables, etc., which they participate in decomposing. The spores pass the winter in the soil. The kind of wine made from grapes depends to some extent on the varieties of yeasts occurring upon them naturally. Yeasts and torulae† may also be found in dust, dung, soil, water and milk, and are not infrequently observed in cultures made with swabbings from the throat. They are also found in insects, flowers, honey, etc. Many species are found as contaminants in brewers' and picklers' vats and many appear to live like bacteria, on the human skin.

**Activities of Yeasts.** Yeasts, especially the very common bakers' and brewers' yeasts, *Saccharomyces*, are characteristically fermentative organisms, producing chiefly alcohol and carbon dioxide from sugar under anaerobic conditions of growth. Their alcohol-forming power is used in the manufacture of wines and beer and was formerly used in production of industrial alcohol. Industrial alcohol is now obtained mainly from the petroleum industry.

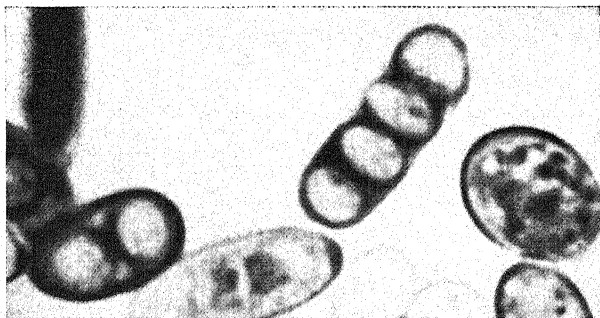


Fig. 3-4. *Saccharomyces cerevisiae* showing asci with two and four ascospores, as well as vegetative cells. (Lindegren, C. C., in Bact. Reviews, vol. 9.)

\* A zygospore is a cell, dormant in character, produced by two similar gametes or reproductive cells. Zygospores are commonly produced by certain algae and molds.

† Asporogenous, yeast-like fungi.



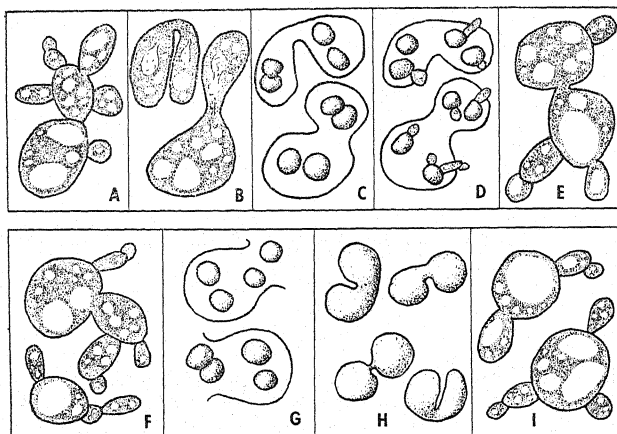


Fig. 3-5. Diagrammatic representation of two types of reproductive cycle in yeast. *A* to *E*, *Zygosaccharomyces* and *Schizosaccharomyces*. *A*. is the vegetative yeast plant, reproducing asexually by budding. The cells in this stage are *haploid*.\* In *B* is seen sexual conjugation of gamete-like cells, with formation of *diploid*† fusion nuclei. In *C* nuclear division occurs, resulting in the formation of haploid ascospores within asci. In *D* the ascospores germinate and grow into the asexually reproducing plant (*E*) again. Growth of these genera is most commonly in the haploid, budding stage.

In *F* to *I* are seen reproductive stages of the common baker's yeast, *Saccharomyces*. *F* shows the asexual, budding plant. *G* shows cells in which budding has ceased and nuclear division occurred, with formation of haploid ascospores which are later liberated from the asci. *H* shows sexual conjugation of the ascospores, with formation of diploid fusion nuclei. After separation these germinate and continue to grow as diploid, asexually-reproducing, budding, yeast plants. This is the usual form of growth of *Saccharomyces*.

Varieties especially adapted to each purpose are used. Thus, there are "distillery yeasts," "top yeasts," for beer, and "bottom yeasts" for beer and wine. Distillery yeasts are the better alcohol producers. The yeasts grow in the beer-wort or fruit juice, utilizing the nutrient substances there. Sterilized beer-wort agar‡ is commonly used for the cultivation of such yeasts.

The carbon dioxide-producing power of yeasts is important in baking. Some yeasts *synthesize* several vitamins, especially those of the B complex. Others *require* a number of vitamins in order to grow and this makes them useful in the assay of vitamins by fermentation methods (see Chapter 44). The cell physiology of yeasts and molds, except as related to reproduction, is basically much like that of bacteria.

#### CLASSIFICATION OF YEASTS

A scheme of possible relations between some groups of common yeasts, molds and fungi is seen in Figure 3-6. The yeasts and some other yeast-like fungi are seen as being derived, by successive loss of filament formation and spore formation, from molds like *Endomyces*. The yeasts may be regarded as

\* In haploid cells the nuclei contain half of the number of chromosomes characteristic of the fertilized or somatic cells of that species.

† In diploid cells the nuclei contain the full number of chromosomes characteristic of the fertilized or somatic cells of that species.

‡ Agar is a vegetable gum often used to prepare solid, jelly-like culture media.

Ascomycetes\* which have lost the property of filament formation. Yeasts and yeast-like fungi are found in two major divisions of the Eumycetes: Class Ascomycetes and Class Fungi Imperfecti (see Table 2).

The yeast-like Ascomycetes are found in the order Endomycetales, and mainly in the family Saccharomycetaceae. There are several genera and many species (see Table 3).

The yeast-like Fungi Imperfecti† comprise several groups which *do not form ascospores*. These fungi are included in the order Moniliales, families Nec-

#### A SUGGESTED SYSTEMATIC RELATIONSHIP OF SOME MOLDS AND YEAST-LIKE FUNGI

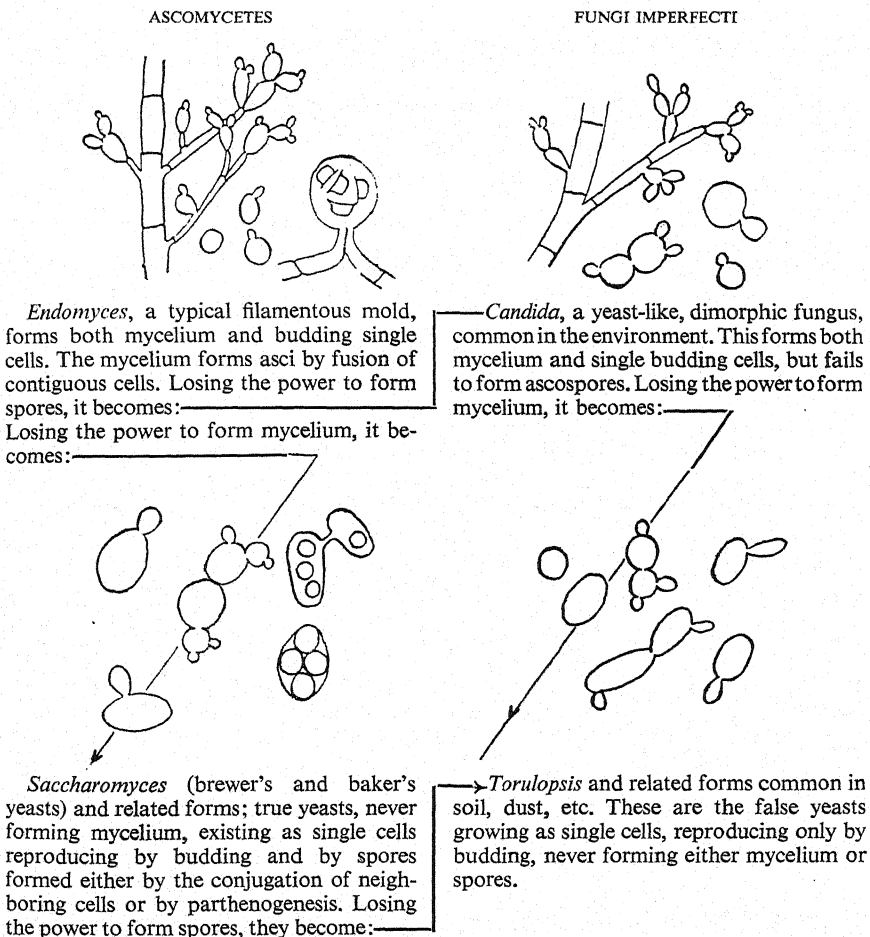


Fig. 3-6. (Reprinted by permission from Henrici, "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., 2nd ed., 1947.)

\* A group of fungi, largely filamentous, in which spores are formed inside of distinctive sacs called *asci* (singular = *ascus*), hence *ascospore* and Ascomycetes.

† Knowledge of life cycle imperfect; especially sexual reproduction.

taromycetaceae and Torulopsidaceae. They are often spoken of collectively as Torulae. They are differentiated from each other on the basis of the presence or absence of asexually produced conidia, pigments, pseudomycelium, form, habitat, etc. (see Table 3).

**Table 3.** *A Classification of Yeasts.*

Class: Ascomycetes (ascospore-forming fungi)

Order: Endomycetales (yeast-like Ascomycetes)

Family Saccharomycetaceae (mycelium scanty or lacking)

(1) Saccharomyceteae

(a) *Saccharomyces*

(b) *Torulaspora*

(c) *Pichia*

(d) *Hansenula*

(e) *Debaryomyces*

(f) *Schwanniomyces*

(g) *Schizosaccharomyces*

(2) Nadsonieae

(a) *Saccharomycodes*

(b) *Hanseniaspora*

(c) *Nadsonia*

Yeasts of commercial importance: some as nuisances, some as valuable aids to bakers, brewers, distillers, commercial alcohol production, wine makers, etc. Some of these species are common in soil, dust, etc.

Class: Fungi Imperfecti (fungi of which the reproductive cycle, especially the sexual stage, is not completely known)\*

Order: Moniliales (conidia, when formed, produced in chains more or less definite)

Family Nectaromycetaceae (form conidia; occur in nectar of flowers)

Family Torulopsidaceae (no conidia; no pigments)

Tribe: Torulopsidoideae (no pseudomycelium)

*Cryptococcus* (one pathogen; mostly saprophytes in soil)

*Pityrosporum* (skin saprophyte)

*Kloeckera* (saprophyte)

*Trigonopsis* (saprophyte)

*Torulopsis* (saprophyte)

Tribe Candidoideae (form pseudomycelium)

*Candida*

*Trichosporon*

Family Rhodotorulaceae (pink and rose-colored pigments)

*Rhodotorula*, etc. (often cause red spoilage of foods, etc.)

\* It has often been pointed out that the imperfections of this group are more in the observations than in the fungi.

**Family Saccharomycetaceae.** The sac-formers, i.e., the ascosporeogenous yeasts, include the tribes Saccharomyceteae and Nadsonieae, in both of which mycelium formation is reduced to a minimum.

THE TRIBE SACCHAROMYCETEAE includes the genus *Saccharomyces*, which is the largest and most familiar group and in which are found most of the common yeasts of commerce, such as *S. cerevisiae* and *S. ellipsoideus*.

The *Saccharomyces* are the most common and valuable industrial yeasts. The cells are oval or elliptical, and oval buds are produced at any part of the cell surfaces. As mentioned above, their fermentative powers are very useful. They are the servants of the baker and brewer. There are numerous species having various special properties useful in industrial processes. Other genera in the tribe are *Torulaspora*, *Pichia*, *Hansenula*, *Debaryomyces*, *Schwanniomyces* and *Schizosaccharomyces*. All of these produce buds at various parts

of the parent cell, i.e., budding is *not bipolar* whereas the cells of the tribe Nadsonieae are characterized by bipolar budding.

THE NADSONIEAE are mainly saprophytic and are of little industrial or agricultural importance.

Genera of Nadsonieae are *Saccharomyces*, *Hanseniaspora* and *Nadsonia*.

The various genera in each tribe may be differentiated from one another by fermentation and other biochemical methods and by the shapes of their ascospores or by their modes of germination or modes of formation of ascospores. Some are found in beer, others in wine vats, dung, soil, etc. (Fig. 3-7).

**The Non-sporeforming Yeasts** (The Fungi imperfecti). There are several subdivisions of yeast-like plants forming no ascospores. A small group, rather specialized as to form and habitat, the Nectaromycetaceae, occurs mainly in honey and insects. The largest group, containing several genera, is called Torulopsidaceae. These organisms closely resemble the yeasts in most respects except that they have not been observed to produce ascospores by any method. They are, therefore, part of the heterogeneous group known as Fungi imperfecti which contains a heterogeneous lot of fungi mainly because their perfect or sexual stage is unknown. The cells of *Torulae* are usually more nearly spherical than are the cells of yeasts. Their classification is not complete. The largest and commonest genus is *Torulopsis*. Various kinds of torula have

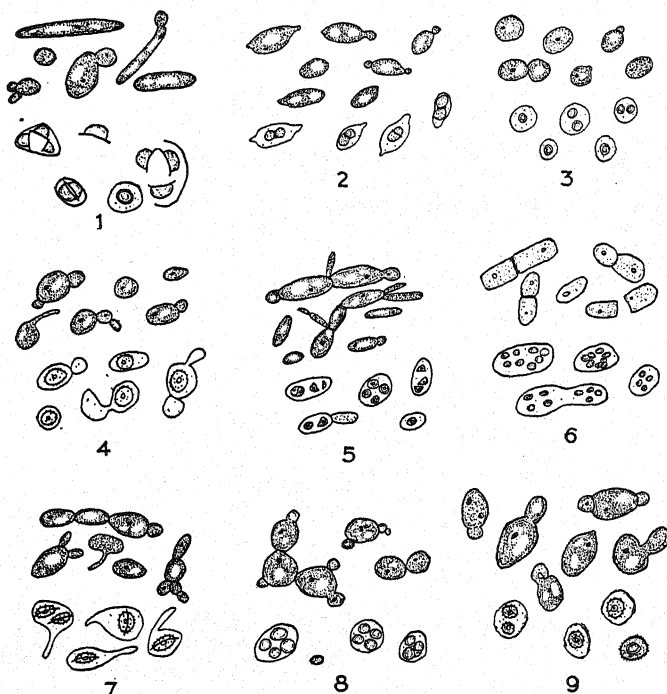


Fig. 3-7. Various forms of yeasts, showing vegetative and budding cells and ascospores. 1. *Hansenula*; 2. *Hanseniaspora*; 3. *Torulasporea*; 4. *Debaryomyces*; 5. *Pichia*; 6. *Schizosaccharomyces*; 7. *Schwanniomyces*; 8. *Saccharomyces*; 9. *Nadsonia*.

been found causing "diseases" of beer and other fermented foods. They are common in soil, water and dust. One species of *Cryptococcus* is pathogenic, infecting the brain and meninges.

Another interesting group, called Rhodotorulaceae, produces brightly colored pigments. Various species in this group, particularly red, pink, or salmon-colored varieties, have been described as agents of spoilage in various organic materials and uncooked foods like canned oysters. They are found as nuisances in places where foodstuffs are prepared, as in butcher shops, and oyster-shucking establishments. They are not pathogenic. Most of the torulas have little fermentative ability and consequently are of little commercial value.

#### REFERENCES

- Alexopoulos, C. J.: *Introductory Mycology*. John Wiley and Sons, New York, 1952.
- Bartholomew, J. W., and Levin, R.: The structure of *Saccharomyces carlsbergensis* and *S. cerevisiae* as determined by ultra-thin sectioning methods and electron microscopy. *J. Gen. Mic.*, 1955, 12:473.
- Connell, G. H., and Skinner, C. E.: The external surface of the human body as a habitat for non-fermenting non-pigmented yeasts. *J. Bact.*, 1953, 66:627.
- de Backze, G. I.: A microbiological process report. Yeasts: I. Morphology. *Ap. Mic.*, 1956, 4:1.
- Etchells, J. L., Bell, T. A., and Jones, I. D.: Morphology and pigmentation of certain yeasts from brines and the cucumber plant. *Farlowia*, 1953, 4:265.
- Hunter, Albert C.: A pink yeast causing spoilage in oysters. *U. S. Dept. Agric. Bull. No. 819*, March 10, 1920.
- Huxley, M. J., and Hurd, R. C.: Pink yeasts isolated from human skin surfaces. *J. Bact.*, 1956, 71:492.
- Lodder, J., and Kreger-Van Rij, N. J. W.: *The Yeasts, a Taxonomic Study*. Interscience Publishers, New York, 1952.
- Scherr, G. H., and Weaver, R. H.: The dimorphism phenomenon in yeasts. *Bact. Rev.*, 1953, 17:51.
- Skinner, C. E., Emmons, C. W., and Tsuchiya, H. M.: *Henrici's Molds, Yeasts and Actinomycetes*. 2nd ed. John Wiley and Sons, New York, 1947.
- White, J.: *Yeast Technology*. John Wiley and Sons, New York, 1954.
- Wickerham, L. J.: Recent advances in the taxonomy of yeasts. *Ann. Rev. Micr.*, 1952, 6:317.
- Wickerham, L. J., and Burton, K. A.: Hybridization studies involving *Saccharomyces* sp., and *Zygosaccharomyces* sp. *J. Bact.*, 1956, 71:290 and 296.

## The Microscopic World

### 4. THE MOLDS

THE TERM "mold" is a convenient one but, strictly speaking, has no exact definition. For present purposes it may be taken to include most of the woolly, cobweb-like, cottony or powdery growth, black, green, yellowish, or white, commonly seen on stale bread, or on old piles of manure, or on books or shoes in the summertime when the humidity is high. We shall also include certain mycelium-producing organisms which have some yeast-like characters. Molds are common on the tops of jams and jellies which have been imperfectly sealed and have stood for a long time. Their spores are as ubiquitous as those of yeasts and bacteria. Molds are able to grow in situations where bacteria cannot survive because of high osmotic pressures, acidity, or low moisture content. They are characteristically strict aerobes and thus cannot compete with anaerobic microorganisms. In general, their metabolic activities are much like those of yeasts and bacteria.

#### STRUCTURE OF MOLDS

The woolly growths consist of more or less compact masses of intertwining, branching, hair-like filaments, called *hyphae*, which grow up into the air. In many species, when circumstances permit, there are also developments of surface or subsurface hyphae on or within the material on which the mold may be growing. These serve to anchor the plant but, except for being more restricted in extent, are not otherwise especially differentiated from the aerial mycelium. The aerial mycelium carries the fertile hyphae which form the reproductive organs while the remainder of the mycelium absorbs moisture and food material.

**The filaments of molds** may consist of elongated cells arranged end-to-end, and separated by walls (*septa*) as in the Ascomycetes. In the Phycmycetes, the whole mycelium consists of one continuous, branching, protoplasm-filled tube with no apparent cross walls. These filaments are said to be *non-septate* or *coenocytic*. In mature plants septation or non-septation is a fairly stable characteristic and serves as an aid in differentiation among molds. As compared with bacteria, the diameter of the filaments of most species of mold is relatively large; sometimes 30 microns.

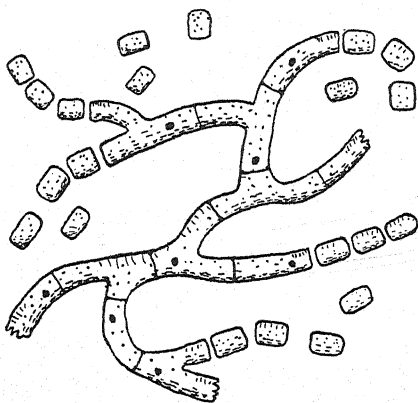


Fig. 4-1. Oidium formation. Segmentation seems to be stimulated so that numerous small cells, often called *arthrospores* are given off. They are not particularly resistant, but serve the purpose of propagation.

The nuclei of molds, within the filaments, are minute, but generally quite definite. In the Ascomycetes (septate) each cell contains one nucleus except during phases of reproduction. In the Phycomycetes (non-septate) the nuclei are not separated by definite cell walls except as a result of the formation of conidia.

The cytoplasm of molds is granular and contains droplets of fat, carbohydrate and nitrogenous material, including volutin. The cell wall of some species probably consists of cellulose; in most, however, the cell wall is made of a chitin-like substance.

As indicated in the table on page 34, molds corresponding to the general description given above may be found in three large divisions of the Eumycetes; namely, Ascomycetes, Phycomycetes, and Fungi imperfecti. Peculiarities of structure, already noted and to be discussed, and methods of reproduction, enable us readily to distinguish filamentous Ascomycetes from Phycomycetes. The Fungi imperfecti will be described later.

#### REPRODUCTION OF MOLDS

Molds reproduce both asexually and (except in Fungi imperfecti) sexually. We may note at least five well-defined methods of asexual reproduction.

1. **Formation of oidia\* or arthrospores.** Septate filaments often form a number of divisions rather closely spaced, resulting in the separation of a number of short, ovoid, cells. These tend to leave the parent filament by *fragmentation*. They continue vegetative growth, each starting a new plant (Fig. 4-1).

2. **Blastospores.** Some of the filamentous molds form buds or *blastospores* along the hyphae. These develop much as do yeasts. Indeed some of these fungi grow readily in either yeast or filamentous form, the form of growth depending on such factors as presence of oxygen and temperature (Fig. 4-2).

3. **Chlamydo spores** are formed by many types of microorganisms. One or more cells acquire thick walls, and become filled with granular reserve material. They probably lose water. In this form they remain dormant and resist drying and sunlight for long periods (Fig. 4-3).

\* Oidium is from a Greek root meaning "egg," i.e., an ovoid body.

4. **Sporangiospores\*** are formed only by the Phycomycetes. They are minute, thick-walled, dehydrated, dormant bodies and are formed in huge numbers by repeated nuclear divisions within globular envelopes called *sporangia*. They are formed at the free ends of fertile hyphae. They resist drying and sunlight for long periods. When the sporangia rupture, the sporangiospores are released. The details of their development and structure are given in the description of the Phycomycetes.

5. **Conidiospores,†** or *conidia*, resemble sporangiospores in many ways. They are formed by Ascomycetes at the free ends of fertile hyphae, but instead of being enclosed in sporangia they are free, sometimes being produced in chains like strings of beads. The form and arrangement of the fertile hyphae and the chains and color of conidia are distinctive of the different genera and species. They are described more in detail in discussing the Ascomycetes.

#### CULTURE MEDIA FOR MOLDS

One of the difficulties in the study of molds and yeasts is their isolation in pure culture. In their natural habitats these microorganisms are practically always intermingled with many other species. A specimen of soil, for example, is inoculated onto a plate of Sabouraud's medium with a view to isolating a particular species of mold in pure culture, i.e., entirely separate from all other microorganisms. In such a situation other molds, yeasts, and especially bac-

Fig. 4-2. A dimorphic, yeast-like, pathogenic fungus (*Candida albicans*) showing mold-like filaments, a few yeast-like cells, blastospores in clusters like grapes near hyphal septa, and circular, thick-walled chlamydo spores. (Photo courtesy of Dr. Lynferd J. Wickerham, U. S. Dep. of Agriculture, Agricultural Research Service.)

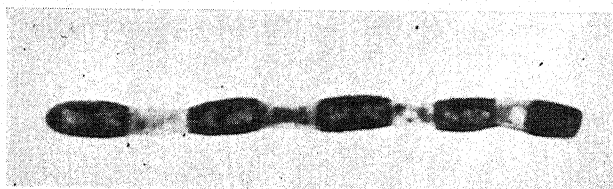
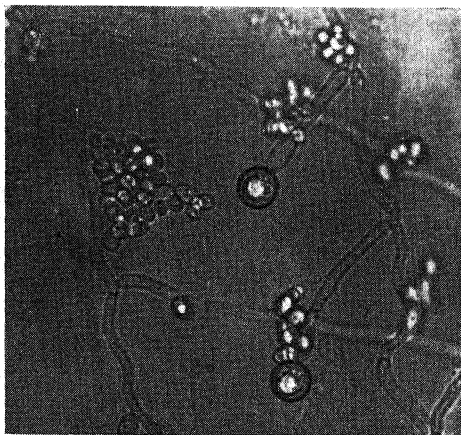


Fig. 4-3. Chlamydo spores. These are formed from mycelial cells by enlargement and by thickening of the cell wall. (Photo courtesy of the U. S. Public Health Service, Communicable Disease Center, Atlanta, Ga.)

\* Angium is from a Greek root meaning bag or envelope; hence, *Sporangium*.

† *Conidio* is from a Greek root meaning dust.



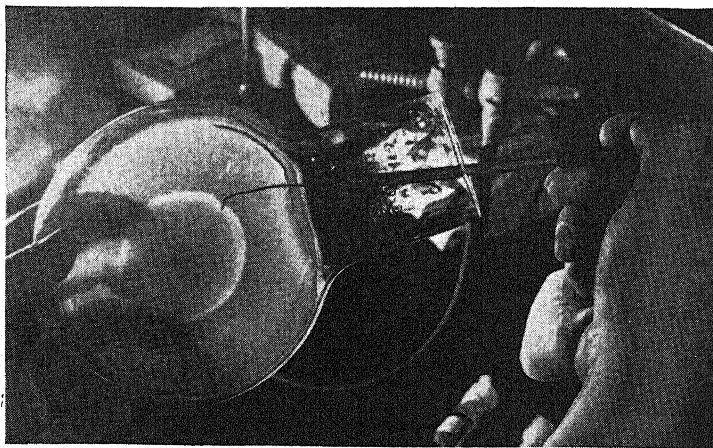


Fig. 4-4. One method of studying fungi in pure culture. A layer of agar medium is allowed to solidify as the flask lies flat. Inoculation then is made in the center of the agar surface with a single touch of an infected needle. After incubation the characters of the single "giant colony" are readily noted. These are distinctive in various species. (Original preparation by Dr. Libero Ajello. Photo courtesy of the U. S. Health Service, Communicable Disease Center, Atlanta, Ga.)

terea which grow much more rapidly than molds, often overgrow and suppress and kill the desired species. To prevent this growth of undesired microorganisms various *inhibitory agents* are used, and various *selective nutrients* which favor growth of the desired species may be used if they are known. For example, a medium especially recommended for isolating fungi from sewage is made of the following: glucose, 10 gm; peptone,\* 5 gm;  $\text{KH}_2\text{PO}_4$ , 1 gm;  $\text{MgSO}_4$ , 0.5 gm; agar, 20 gm; water, 1000 ml; rose bengal, 10.035 gm; streptomycin, 30.0  $\mu\text{g}$ † per ml. Rose bengal is a dye which inhibits growth of many bacteria. Streptomycin, an antibiotic, also inhibits many unwanted organisms. The glucose and peptone furnish organic carbon and nitrogen foods as well as organic sources of energy. The  $\text{KH}_2\text{PO}_4$  maintains a suitable degree of acidity (pH) while  $\text{MgSO}_4$  furnishes magnesium and sulfur; both essential to the formation of protoplasm.

Other combinations of inhibitory agents like oxgall, crystal violet and streptomycin have been found very useful. One containing cycloheximide, streptomycin, and penicillin is particularly valuable in medical mycology. Cycloheximide is an antibiotic which, curiously enough, inhibits most common, air-borne, *saprophytic* molds which contaminate medical specimens, but permits the *pathogenic* species, with very few exceptions, to multiply freely.

Among the most distinctive properties of molds are morphology of mycelia, sexual and asexual reproductive mechanisms, pigments and manner of colony growth.

**Giant Colonies.** An excellent method of demonstrating gross morphological details, pigment, etc., is in the form of giant colonies. Appropriate culture media especially designed to favor development of reproductive mecha-

\* Partly digested protein.

†  $\mu\text{g}$  is the symbol for microgram: 1 millionth of a gram.

nisms, pigments, etc., are used. For example, thiamine-enriched casein agar is especially useful in stimulating conidia formation in certain skin-infecting fungi (*dermatophytes*). Whatever medium is selected, it is poured into plates, flasks or bottles of about 200 ml capacity, to a depth of about  $\frac{1}{3}$  inch, sterilized and allowed to solidify. This gives a broad surface and the volume of agar serves as a reserve of moisture. A speck of inoculum (pure culture) is transferred to a point in the center of the agar. It is well to do this with the bottle or flask in an inverted position to avoid contamination by dust. Plugged with cotton, the flask is held at appropriate incubating temperature for some days. Giant colonies grow out, showing various details of color, form, etc., characteristic of the different organisms. A colony of this kind is seen in Figure 4-4. Giant cultures may be examined with low power lenses *in situ*, or portions may be teased out on a slide in a drop of mounting fluid\* and examined under a cover slip. Drying produces shrinkage and distortion, while manipulation, such as teasing out, causes fracturing and loss of conidia. It is much better to examine growth *in situ* with the microscope.

**Slide Cultures.** For the microscopic examination of molds the slide culture is an excellent method. A good arrangement is to cement a sterile cover slip to an ordinary (previously sterilized) slide with two other bits of glass arranged so that the cover slip is raised about 1 millimeter above the slide. The desired nutrient agar, melted, cooled to about 45° C. and inoculated with spores, is admitted between the slide and the cover slip and the culture incubated (Fig. 4-5). This permits examination of the growth with fairly high power lenses

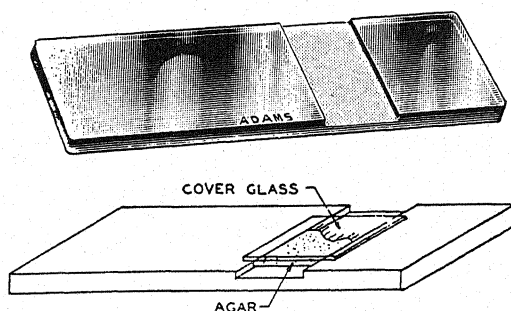


Fig. 4-5. Microscope slide arranged for small cultures of molds, etc. Agar of appropriate composition, mixed, while still fluid, with spores or parts of the desired fungus, is allowed to run under the cover slip. Growth occurs at the margin under the cover slip. Microscopic examination of the fungus as it grows is thus possible. (Courtesy of Dr. Richard N. Shoemaker, Science, 1950, vol. 112.)

\* An excellent mounting fluid is lactophenol-cotton blue solution, made as follows:

	ml
Phenol crystals (melted).....	20
Lactic acid.....	20
Glycerin.....	40
Dist. H <sub>2</sub> O.....	20

Dissolve by heating mixture at about 70° C. Add 0.05 gm of dye called Poirrier's blue or cotton blue.

A drop of this solution is placed on a slide and a small part of a fungus colony is teased apart in it with needles. A thin cover slip is placed over it, and the preparation is examined with the microscope.

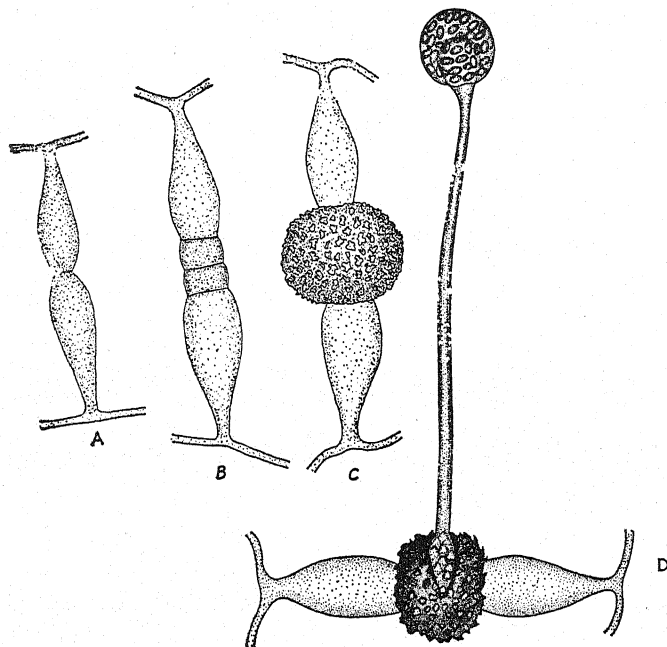


Fig. 4-6. Zygospore formation (sexual reproduction) by *Rhizopus*. *A*, two swollen hyphae grow toward each other and meet. *B*, each produces a special cell (gamete). These then fuse or conjugate. The resultant fertilized cell develops into a dormant zygospore (*C*) which later germinates, sending out a hypha which produces conidia asexually (*D*). (From Swingle, D. B., *Plant Life*, 2nd ed. D. Van Nostrand Co., Inc.)

and in a living, undisturbed condition. This slide may be placed in a covered Petri dish with a bit of moist cotton.

The best conditions of incubation for most molds are a moist atmosphere and a temperature around 30° C. There should be free access of oxygen. For general purposes the best pH is around 6.5-7.0. Other slide-culture techniques have been described by several mycologists.

#### HABITAT AND GENERAL PROPERTIES OF MOLDS

Mold conidia are found in all dust as every housewife knows who has had preserves spoiled by molds. Molds occur on decomposing organic matter like manure piles, "compost" heaps, dead plants and animals. The sea has a most interesting, though somewhat limited, indigenous flora of molds. They participate in the destruction of ropes, timbers, etc., exposed at water level. Some have done enormous damage by infecting commercially valuable fish, shell-fish, and animal and fish foods (such as "eel grass") on which many edible marine forms live.

Molds decompose cellulose and ruin paper and wood products not protected from them. They grow in and under paint on walls and cause flaking and deterioration of the paint. They grow on the surfaces of lenses in binoculars, etc., in the tropics, diminishing clarity of vision. They also grow on the surface of electrical insulators, causing them to transmit electricity. Some

molds grow well on rubber and ruin it. Molds are merry jokers of the microscopic world!

Physiologically, molds are, as a group, active in causing very rapid oxidations and decompositions of carbohydrates, fats and proteins, and other biochemical changes in a great variety of substances. They are of great commercial value in the production of various organic compounds which are used as foods, flavors and drugs. From carbohydrates they produce hundreds of substances not easily made by artificial processes, and of great usefulness. Among these are penicillin, etc., acetone, butanol, sorbitol, takadiastase, and so on. This is an excellent field for research.

### THE PHYCOMYCETES

The group of Phycomycetes contains several genera, two of which are very commonly observed and often very troublesome, namely *Mucor* and *Rhizopus*. These are placed in a group called Zygomycetes because they form *zygospores* (see Fig. 4-6). One aquatic genus of Phycomycetes, called *Saprolegnia*, is parasitic on fish.

**Genus Mucor.** This genus contains several dozen species, many of them

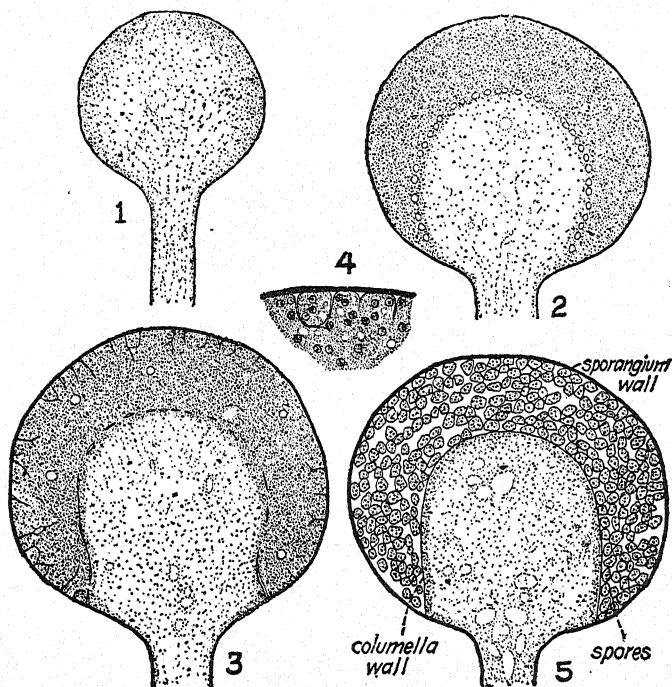


Fig. 4-7. One of the Mucorales. Stages in formation of a sporangium filled with spores. 1, sporangiophore and columella; 2, multiplication of spores from nuclei of the columella and formation of the retaining membrane; 3, completion of sporangium formation; 4, beginning of segregation of multiplied nuclei to form the individual spores or conidia; 5, final structure of the sporangium showing the thickened retaining membrane, the free conidia ready for dispersion on rupture of the membrane, and the now inert, rounded columella which will be left behind. (From Swingle's Plant Life, D. Van Nostrand Co., Inc.)

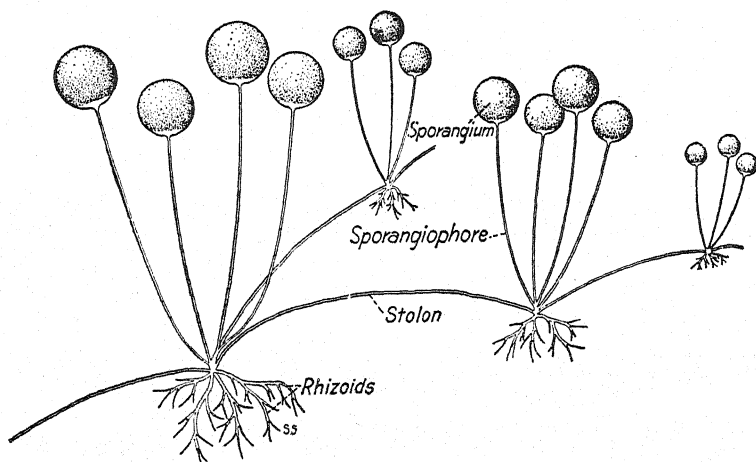


Fig. 4-8. *Rhizopus nigricans*. Plant spreading by stolons and rooting itself by means of rhizoids. (From Swingle's Plant Life. D. Van Nostrand Co., Inc.)

very similar to one another. One of the best known is *Mucor mucedo*, a coarse, woolly, white mold seen on piles of rotting manure or other decaying organic matter. *Mucor mucedo* reproduces asexually by means of sporangiospores. Each sporangium is borne on a short hypha called a *sporangiophore*\* at the tip of which is an enlarged portion called a *columella* which varies in shape according to the species. The spores are formed in a mass about the columella within the envelope (Fig. 4-7).

In sexual reproduction zygospores are formed (Fig. 4-6). The cells fusing to form zygospores are usually on two *separate* plants, which produce the spores wherever hyphae come into contact, suggesting that the plants are of opposite sexes. Neither plant alone produces zygospores. Such plants are said to be *heterothallous*, and their "sexes" are called + or -. When hyphae of the same plant fuse, the plant is said to be *homothallous*.

Mucors and related genera of molds are common contaminants of bacteriological cultures. A few have been found as the cause of disease in man and animals.

**Genus *Rhizopus*.** The molds of this genus, all typical Phycomycetes, are well exemplified by *R. nigricans*, the common, black, bread mold familiar to all who have seen bread after it has stood in a humid place for some days during the summer. It spreads rapidly because it sends out stolons or runners (Fig. 4-8) like some kinds of grass ("Bermuda grass") and strawberry plants. These runners take hold of the substrate by means of "holdfasts" or root-like hyphae.

Like *Mucor mucedo* and its allies, *R. nigricans* is prominent in bringing about decomposition and spoilage of various fruits, vegetables, and other organic materials.

Molds of the genera *Mucor* and *Rhizopus* may be differentiated by at least three characters as follows:

\* A stalk on which the sporangium is borne.

	<i>Mucor</i>	<i>Rhizopus</i>
Runners.....	Absent	Present
Sporangiophores.....	May arise at any point in the mycelium.	Arise only at the "holdfasts" where the runners touch the substrate.
Columella.....	Never hemispherical; continuous with the sporangio-phore.	Hemispherical; a single, differentiated cell.

### THE FILAMENTOUS ASCOMYCETES

We have already encountered sac-forming fungi (Ascomycetes) as yeasts. We now review the filamentous Ascomycetes.

The group of Ascomycetes as a whole contains many widely differing plants, varying from the yeasts through filamentous wooly molds, to fleshy mushroom-like morels and some of the beautifully formed and colored fungi which grow on rotting logs. All have in common the formation of asci with ascospores and all are modifications, or evolved and elaborated patterns, of the same basic growth design. They have septate mycelia.

Among the best known Ascomycetes are the common genera *Aspergillus* and *Penicillium*. These are distinguished from Phycomycetes (like *Mucor* and

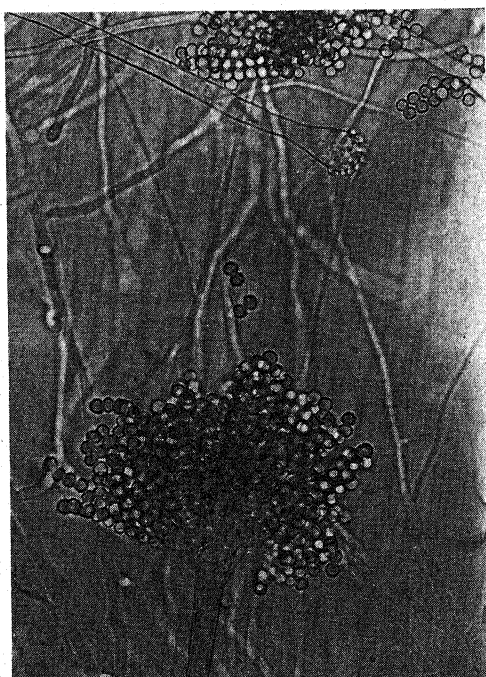


Fig. 4-9. *Aspergillus* sp., showing typical structures: near upper left a fertile hypha or conidiophore arises by branching almost at a right angle from a short hyphal cell called a foot cell; near low center is seen a large conidiophore, from the rounded tip (vesicle) of which arises a cluster of spindle-shaped supporting cells called sterigmata; from the sterigmata arise chains of spherical conidiospores. A denuded conidiophore is seen near the top of the picture. (Photo courtesy of Dr. Lynferd J. Wickerham, U. S. Dep. of Agriculture, Agricultural Research Service.)



Fig. 4-10. A perithecium of *Allerscheria boydii*, a pathogenic, filamentous ascomycete occurring in the soil, has matured and ruptured, liberating many ascospores. (Courtesy of Dr. Libero Ajello, in *Am. J. Trop. Med. and Hyg.*, 1952, vol. 1.)

*Rhizopus*) by (1) forming numerous sexual spores in sacs (ascospores); (2) producing conidiospores in groups of various sorts such as chains, masses of characteristic shape, etc., (Fig. 4-9); and (3) being septate.

**Genus *Aspergillus*.** During sexual reproduction (rarely seen) hollow structures called *perithecia*, which contain *asci* with spores, are formed. It is for this reason that the aspergilli are included in the same order (Ascomycetes) as the yeasts. Both form ascospores.

**PERITHECIUM FORMATION.** In the formation of a perithecium two hyphal cells twist about each other. The separating walls dissolve and the resulting fusion-cell gives rise to a nodule of branched hyphae among which a mass of asci is formed. The whole mass is often enclosed in a sort of hull or protective cellular covering formed by the surrounding mycelium (Figs. 4-10, 4-11).

**THE ASEQUAL FRUITING BODIES**, or conidiophores, of aspergilli consist of tall hyphae arising as a branch of a cell in the mycelium called a "foot-cell." The fruiting hyphae have enlarged globular tips (vesicles). From the surface of these numerous small stems, called *sterigmata*, radiate in all directions. On the tips of these the conidiospores are borne in long chains (Fig. 4-9). There is no sac or envelope as in the Zygomycetes. Classifications of aspergilli are based to some extent on the structure, but more particularly on the color, of the conidial heads.

**COMMON SPECIES.** One of the commonest species of *Aspergillus* is *A. glaucus*. It forms green or grey-green conidia and yellow perithecia and is frequently seen on bread, preserves and clothing during the summer or in the tropics. Another common environmental species, sometimes found as a laboratory nuisance, *A. nidulans*, is recognized because of its bright green color. The perithecia are reddish in color. *A. niger*, also an extremely common

species, is recognizable by its very large round masses of black conidia. It is sometimes confused with *Rhizopus nigricans* which produces black sporangia.

There are some pathogenic species. For example, a pulmonary infection of birds due to *Aspergillus fumigatus* is not uncommon.

**Genus *Penicillium*.** The penicillia are widely distributed and contribute to the spoilage of various objects and materials composed of organic matter, especially ripe fruits. The conidiophores are composed of hyphae which branch at the tip into fingerlike clusters, the whole roughly suggestive of the bony structure of the hand. The spores extend in chains from the ends of the "fingers" (sterigmata). This arrangement gives the whole conidiophore with its chains of conidiospores a form suggestive of a tiny paint brush, from which the generic name is derived (Fig. 4-12, B). As in other groups of molds, the color and form of the fruiting body furnish characters of differential value in classification. Sacs or perithecia are formed by some penicillia in much the same manner as by the aspergilli, but most species of *Penicillium* are classed as Fungi imperfecti.

Some species of *Penicillium* are differentiated chiefly by their habitat. Thus, the green molds found in Roquefort cheese (*P. roqueforti*), Camembert cheese (*P. camemberti*) and other cheeses of the same nature are distinguished chiefly because of their occurrence there. The molds grow in or on the cheese, producing various enzymatic changes in the fat, carbohydrate and protein of the cheese which result in characteristic aromas, flavors and textures. *P. roqueforti* grows well under conditions of temperature and humidity found in the limestone caverns in the province of Roquefort, France. It is micro-aerophilic, growing in the interior of the cheese masses provided holes are punched in the cheese to admit small amounts of air. (These holes must not be confused with the holes in Swiss cheese produced by gas formed by the *Propionibacter*.)

*Penicillium camemberti*, which closely resembles *P. roqueforti*, is strictly aerobic and grows only on the exterior of Camembert cheese, so that the ripening of this delicacy proceeds from without inward.

There are many other species of penicillia and they are frequently seen on old bread, cheese, lemons, and other fruits. They may usually be recognized as members of the genus by their sky-blue or green color. The individual

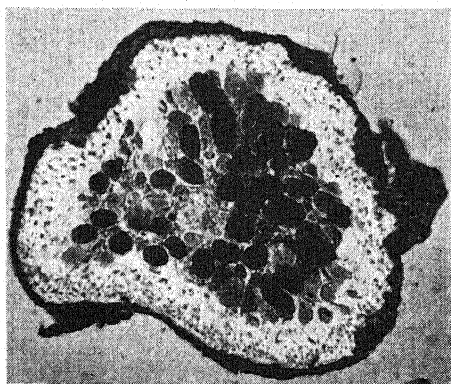


Fig. 4-11. Structure of a perithecium of recently discovered *Westerdykella ornata*, an East African ascomycete. Note the thick perithecial wall and the contained asci, each having 32 ascospores inside. ( $\times 200$ ). (Courtesy of Dr. Amelia C. Stolk; in Trans. Brit. Mycolog. Soc., 1955, vol. 38.)



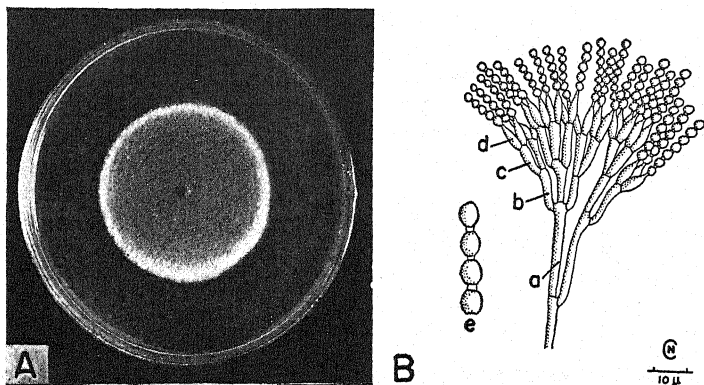


Fig. 4-12. *A*, *Penicillium* sp. The rapidly growing giant colony (about  $\frac{1}{2}$  life size) is at first white, then becomes bluish-green and very powdery due to abundant conidia or spore production from the aerial mycelium. *B*, *Penicillium* sp. Spore-bearing hyphae characteristically form a "penicillus" (Latin for brush). The conidia occur in unbranched chains (*e*) formed at the tips of flask-shaped sterigmata (*d*) which are verticillately arranged (in whorls) from the ends of metulae (*c*) arising from branches (*b*) of the conidiophore (*a*). Although species of *Penicillium* differ in gross appearance (size of colony, color, texture, etc.), the genus may be identified by the characteristic structure of the conidiophore and penicillus. (Conant, Martin, et al., *Manual of Clinical Mycology*.)

*species* may be differentiated by the various arrangements of the conidiophores.

*Penicillium notatum* and *P. chrysogenum*, very similar species, have come into great prominence as sources of penicillin.

#### FUNGI IMPERFECTI

Fungi imperfecti comprise nonphycomycetous (septate) fungi of which the sexual reproductive cycle is not known. The grouping is wholly artificial. The membership of the group is, therefore, rather heterogeneous since imperfect forms of Ascomycetes, Basidiomycetes, etc., are numerous. Most Fungi imperfecti, however, are really Ascomycetes of which our knowledge is incomplete.\* Many of the species of Fungi imperfecti probably have complete sexual cycles if we only knew the proper kind of medium and growth conditions which would permit their development. The imperfection lies not so much in the fungi as in our knowledge!

Since there are about 1,330 genera and some 20,000 species, a complete discussion of the Fungi imperfecti here is out of the question. We may, however, give attention to a few common species of filamentous fungi of this group which any bacteriologist may from time to time encounter in his laboratory work.

**Genus *Trichoderma*.** The conidia of this genus occur in compact masses on tips of well-developed conidiophores which branch like trees or shrubs. They are bright green in color. *Trichoderma* is common in soil and as a contaminant in laboratory cultures of the microbiologist.

The best-known species of this genus is *T. koningi* or *T. viride*, a common

\* Most of the *Aspergillus* and *Penicillium* species are Fungi imperfecti. Since these genera have already been described they are not discussed here.

soil and environmental species which often enters laboratory cultures unin-  
vited (Fig. 4-13). It forms large amounts of ammonia, and an ammoniacal  
odor gives a clue to its identity.

**Genera Candida, Trichosporon, Geotrichum.** These three genera are sub-  
divisions of a large group sometimes called *Oidium*, sometimes *Monilia*,\* and  
often *Candida*. In the main, these organisms are neither clearly filamentous  
molds nor are they exclusively yeast-like, but partake of the characters of  
both. The yeast-like characters are more often seen.

The group may be divided into three genera based upon mode of repro-  
duction: (1) *Geotrichum*, reproducing by fragmentation or disarticulation of  
the mycelium into separate cells called *arthrospores*; (2) *Candida*, reproducing  
by development of yeast-like cells budding from the ends or sides of the  
mycelium, called *blastospores*; (3) *Trichosporon*, reproducing by both methods.

**GEOTRICHUM.** One common species is the usually harmless *Geotrichum*  
*candidum*, often called *Oidium lactis*. It is a saprophyte of soil, etc., and is  
often found in the oral cavity of normal persons. It can cause serious infections  
under some conditions. This species readily metabolizes lactic acid and is of  
common occurrence in soured dairy products, like sour milk, cheese or butter,  
and in sauerkraut and silage, where lactic acid is formed by other organisms.  
It grows as a white, felt-like, adherent membrane on the surface of solid  
media like Camembert and other cheeses, clotted milk, or agar (Chapter 44).  
Aerial mycelium forms as the plant grows older.

**CANDIDA ALBICANS** is commonly found on the skin or on the oral or vaginal  
mucous membranes or in the feces of normal individuals. It also occurs in  
soil, dung, etc. Growth on Sabouraud's agar is best at 37° C. The colonies are  
creamy and have a yeasty odor. The growth consists mainly of small (2 to  
4  $\mu$ ), yeast-like cells. Mycelial elements may occur, with blastospores. The  
formation of clusters of budding cells and distinctive, round chlamydospores  
on cornmeal agar or other suitable agar are used in identification. (Fig. 4-2.)

This species can cause a number of serious infections of man and animals,  
especially of mucous membranes (*thrush*, *vulvovaginitis*, etc.), and the skin  
(*cutaneous candidiasis*) and lungs (*pulmonary candidiasis*). Microscopic ex-  
aminations of scrapings from lesions usually reveals the organisms but diag-  
nosis should be made only by a skilled mycologist, especially as these and  
similar species are so common in normal individuals.

**Genus Alternaria.** Several kinds of dark-green or brownish-green molds  
from soil and dust are often met with as contaminants in laboratory media

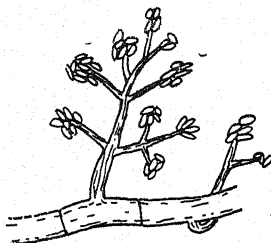


Fig. 4-13. *Trichoderma koningi*. (Re-  
drawn from Henrici.)

\* Monile is from the Latin for necklace; a string of beads.

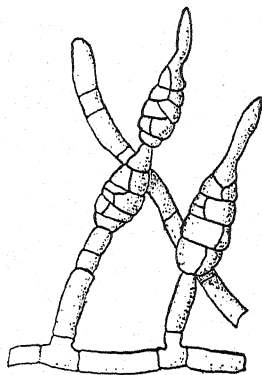


Fig. 4-14. *Alternaria*, showing muriform multicellular conidia.

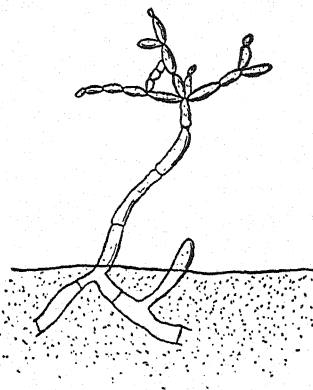


Fig. 4-15. *Hormodendrum*; dark, smoky or black colonies.

and on Petri-dish cultures. Among the commonest forms are members of the genus *Alternaria*. They are not so large and extensive as *Aspergillus* or *Penicillium*. The colonies of *Alternaria* are woolly but are more compact, and the underside and mycelium are very dark colored. The conidia are relatively large, and their 8 to 14 or more large, component cells occur packed in distinctive, muriform,\* conical masses so that the cells are irregularly shaped. These groups of conidia are often arranged in long rows, or occur along the mycelial filaments (Fig. 4-14).

**Genus *Hormodendrum* or *Cladosporium*.** These molds, like *Alternaria*, form relatively small, dark-green or brownish-green colonies with a fine-textured, velvety surface. They produce conidia by growth at the *tip* of the chains (*apically*) rather than at the base of the chains as in *Aspergillus* and *Penicillium*. Thus, in contrast to the last two, the youngest conidia in *Hormodendrum* are at the tips. The conidia are rather oval or elongated, and the chains of conidia are often branched (Fig. 4-15).

#### SOME PATHOGENIC FUNGI

***Blastomyces dermatitidis***, also sometimes called *Cryptococcus gilchristi* or *Oidium dermatitidis*, is a dangerous pathogen, causing the disease known as North American blastomycosis. *B. dermatitidis* is one of the dimorphic,

\* Muriform is from the Latin word *murus* (wall) meaning "arranged like bricks in a wall."

yeast-like fungi capable of invading superficially or throughout the whole body. In tissues and in cultures at 37° C only very thick-walled, budding, yeast-like cells occur. In cultures at 22° C mold-like filaments occur.

A very similar organism, called *Cryptococcus neoformans*, causes a similar disease known as "European" blastomycosis, but world-wide in distribution. It does not form filaments in cultures but is distinguished by its very large, gummy capsules.

***Coccidioides immitis*** is the cause of a disease called *coccidioidomycosis*, occurring chiefly in the San Joaquin Valley of California and elsewhere in arid regions. In nature the organisms live in the soil and their resistant chlamydospores and arthrospores from mycelial filaments are blown about with dust and inhaled. Many of these infections pass unnoticed or result in a febrile disease in association with bronchitis, "rheumatism," or pneumonia. The disease is often confused with tuberculosis in x-ray and clinical examinations.

When *C. immitis* is specially cultivated away from free oxygen, or when invading the tissues of the body, it forms cells resembling those of *B. dermatitidis*, but they never form buds. On the contrary, the cell contents divide into many smaller cells, within the cell wall. The sporangium wall ruptures, liberating large numbers of small cells. These are then distributed by the blood throughout the body and repeat the cycle. When cultivated aerobically, or in the soil, mycelial filaments are generally formed. Thus, it is a dimorphic fungus. No conidia are formed (Fig. 4-16).

***Histoplasma capsulatum***. This organism resembles *B. dermatitidis* and *Coccidioides immitis* in several respects. It causes infections in man which apparently may pass unnoticed in certain individuals, yet may cause fatal generalized infections in other persons. The infection (called *histoplasmosis*) appears to be widespread in the Ohio, Mississippi and Missouri river basins and elsewhere all over the world. These infections are pulmonary in character, in some respects resembling tuberculosis. The infection has been found naturally occurring in dogs, rats, skunks and cattle, soil (another soil saprophyte as a pathogen), and dust of the air.

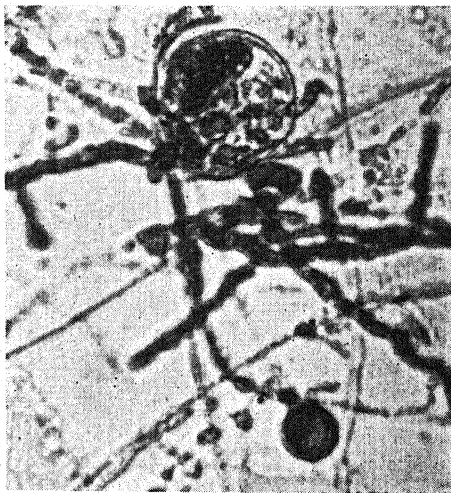


Fig. 4-16. *C. immitis* stained with lactophenol cotton blue. Filaments, probably with arthrospores, are seen as well as a germinating sporangium or spherule. (Burke, R. C., Proc. Soc. Exp. Biol. & Med., 1951, vol. 76.)

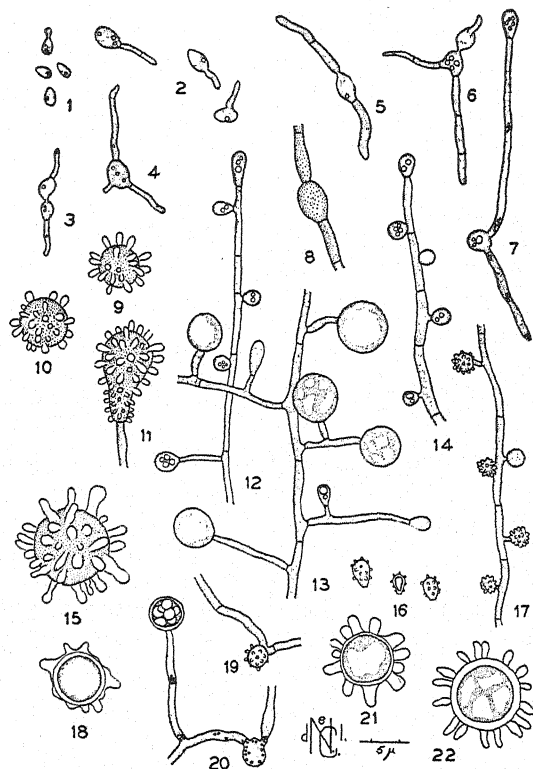


Fig. 4-17. Life cycle of *Histoplasma capsulatum*. 1, yeast cells from blood agar incubated at 37° C. 2, yeast cells germinating with a single tube in 48 hours in Sabouraud's glucose agar culture at room temperature. 3, yeast cells with tube germinating from each cell on Sabouraud's glucose agar. 4, single yeast cell with three germ tubes on Sabouraud's agar. 5, single yeast cell with two germ tubes on Sabouraud's agar. 6, budding yeast cell which has germinated with three tubes on Sabouraud's agar. 7, 20, smooth-walled chlamydospores which have germinated in Sabouraud's broth and produced chlamydospores in eight days. 8, intercalary chlamydospores on Sabouraud's glucose agar. 9, 10, 15, round tuberculate chlamydospores from Sabouraud's glucose agar. 11, Pyriform tuberculate chlamydospore from Sabouraud's agar. 12, 14, small, round, smooth-walled sessile and terminal chlamydospores with oil droplets. 13, large round smooth-walled chlamydospores on Sabouraud's glucose slide culture in fourteen days. 16, small pyriform tuberculate chlamydospores from Sabouraud's glucose agar. 17, small round tuberculate chlamydospores on aerial hyphae. 18, 21, 22, optical view of thick-walled chlamydospores. 19, small tuberculate chlamydospore germinating in cell culture. (Conant, N. F. in *J. Bact.*, vol. 41.)

This organism may be cultivated on 10 per cent blood infusion agar at 37° C. Under such conditions the cells are yeast-like. In infected tissues only the yeast-like form is seen.

When cultivated at room temperatures on such media as Sabouraud's or glucose agar, a cottony, white, filamentous growth appears, another dimorphic fungus. The filamentous form is septate and in older cultures there appear large, round, or pear-shaped, thick-walled chlamydospores covered with rounded projections (*tuberculate* chlamydospores) (Fig. 4-17). These are especially characteristic of *H. capsulatum*.

## THE DERMATOPHYTES

Mycotic (fungal) infections of the skin are common; in fact skin mycoses are among the commonest of infectious diseases. The molds most commonly involved are a rather well-demarcated group called *dermatophytes*. In general they belong to the Fungi imperfecti.

Attempts to classify the dermatophytes have left the taxonomy and nomenclature of this group in a somewhat confused state. For convenience we may list some pathogenic fungi as follows:

## I. YEAST-LIKE FORMS

- Torulopsis* (meningitis; European blastomycosis)
- Candida* (thrush; moniliasis; paronychia; vaginitis)
- Blastomyces*

## II. FILAMENTOUS FORMS

## A. Ringworm (tinea) fungi.

1. *Microsporium*
  - M. canis* (animal type; tinea capitis in children)
  - M. audouinii* (human type; tinea capitis in children)
  - M. gypseum* (animal type; tinea)
2. *Trichophyton*
  - T. schoenleini* (favus)
  - T. mentagrophytes* (kerion; tinea sycosis)
  - T. rubrum*; *T. megnini*; *T. violaceum* (various forms of tinea, kerion, etc.)
  - T. tonsurans* ("barber's itch" or tinea capitis)
3. *Epidermophyton*
  - E. floccosum* (mycosis of hands, feet and groin. Similar conditions caused by various *Trichophyton* species)

## B. Other pathogenic filamentous fungi.

1. *Coccidioides immitis*
2. *Sporotrichum*

## REFERENCES

- Ajello, L.: Soil as a natural reservoir for human pathogenic fungi. *Science*, 1956, 123:876.
- Benham, R. W.: The genus *Cryptococcus*. *Bact. Rev.*, 1956, 20:189.
- Conant, N. F., Martin, D. S., Smith, D. T., Baker, R. D., and Callaway, J. L.: *Manual of Clinical Mycology*. 2nd ed. W. B. Saunders Co., Philadelphia, 1955.
- Cooke, W. B.: Fungi in polluted water and sewage: I, II, III. *Sewage and Ind. Wastes*, 1954, 26:539, 661, 790.
- Cutter, V. M., Jr.: The cytology of fungi. *Ann. Rev. Microbiol.*, 1951, 5:17.
- De Yay, J. E.: Mutual relationships in fungi. *Ann. Rev. Micr.*, 1956, 10:115.
- Emerson, R.: *Molds and Men*. *Sci. Amer.*, 1952, 186:28.
- Georg, L. K.: The role of animals as vectors of human fungus diseases. *Tr. New York Acad. Sci.*, 1956, Ser. II, 18:639.
- Georg, L. K., Ajello, L., and Papageorge, C.: Use of cycloheximide in the selective isolation of fungi pathogenic to man. *J. Lab. Clin. Med.*, 1954, 44:422.
- Gordon, M. A., and Cupp, H. B., Jr.: Detection of *Histoplasma capsulatum* and other fungus spores in the environment by means of the membrane filter. *Mycologia*, 1953, 45:241.
- Haley, D. L.: Culture media used in a diagnostic medical mycology laboratory. *Am. J. Med. Technol.*, 1954, 20:4.
- Hotchkiss, M.: Methods for the isolation of pathogenic fungi from clinical material. *Am. J. Med. Technol.*, 1953, 19:154.
- Lilly, V. G., and Barnett, H.: *Physiology of the Fungi*. McGraw-Hill Book Co., Inc., New York, 1953.
- Littman, M. L., and Zimmerman, L. E.: *Cryptococcosis (Torulosis)*. Grune and Stratton, New York, 1956.

- O'Hern, E. M., and Henry, B. S.: A cytological study of *Coccidioides immitis* by electron microscopy. *J. Bact.*, 1956, 72:632.
- Raper, K. B., and Thom, C.: *Manual of the Penicillia*. Williams & Wilkins Co., Baltimore, 1949.
- Raper, K. B., and others: Speciation and variation in asexual fungi. *Ann. New York Acad. Sci.*, 1954-55, 60(Art. 1):1.
- Reiss, F., and others: Medical Mycology. *Ann. New York Acad. Sci.*, 1948-1950, 50:1209.
- Ritchie, D.: A fungus flora of the sea. *Science*, 1954, 120:578.
- Skinner, C. E., Emmons, C. W., and Tsuchiya, H. M.: *Henrici's Molds, Yeasts and Actinomycetes*. 2nd ed. John Wiley and Sons, New York, 1947.
- Smith, C. E., (Chairman), *Proc. Conf. on Histoplasmosis*. Pub. Health Serv. Publ'n. No. 465, 1956. Gov't. Printing Off., Washington 25, D.C.
- Smith, G., and Raistrick, H.: *An Introduction to Industrial Mycology*. Edward Arnold & Co., London, 1942.
- Thatcher, F. S.: Foods and feeds from fungi. *Ann. Rev. Microb.*, 1954, 8:449.
- Thom, C., and Raper, K. B.: *A Manual of the Aspergilli*. Williams & Wilkins Co., Baltimore, Md., 1947.
- Vishniac, H. S.: Marine mycology. *Tr. New York Acad. Sci.*, 1955, Ser. II, 18:352.
- Wolf, F. A., and Wolf, F. T.: *The Fungi*, Vols. I and II. John Wiley and Sons, New York, 1947.

## The Microscopic World

### 5. THE VIRUSES.\* A. GENERAL DISCUSSION

✓ IN 1891, BACTERIA were the smallest, simplest and lowest forms of life known. Physiologically and structurally they were viewed as the boundary between the living and the inanimate. Investigators of that time felt that they had probed the depths of the mystery of life and discovered its extreme lower limit with respect to size and simplicity of organization. Yet many times the clear, colorless, and seemingly sterile fluids through which their searching lenses swept, teemed with billions of living particles which escaped their vision and their knowledge.

#### FIRST DEMONSTRATION OF FILTRABLE INFECTIOUS AGENTS

✓ **Plant Viruses.** In 1892, Iwanowski demonstrated that a disease of the tobacco plant called "tobacco mosaic" could be transmitted to healthy plants by the sap from diseased plants after the sap had been passed through filters of porcelain so fine as to remove all bacteria. No living thing grew from the sap of diseased plants on any culture media in the laboratory and nothing could be seen in the crystal-clear fluid with a microscope. But we now know that the sap from the diseased plant contained millions or billions of particles of the virus of tobacco mosaic; the first-known viral disease of plants. Iwanowski had opened the door to the world of the ultramicroscopic, much as Leeuwenhoek's discoveries had opened the door to the world of the microscopic.

✓ **Bacterial Viruses.** The group of viruses which attack bacteria was first described in 1915 by the British scientist Twort and more fully studied about 1917 by the French investigator, d'Herelle. d'Herelle named these viruses bacteriophage.† Bacteriophage is one of the most interesting and important living organisms, as will be seen farther on in this discussion.

\* The word *virus* is derived from a Latin root meaning a slimy, poisonous liquid (e.g., snake venom). Today our meaning of the word virus is somewhat different but not much more specific.

† The word bacteriophage is derived from the word *bacterium* and a Greek root *phagein*, meaning to eat. The bacteriophage was originally thought of as eating bacteria from within. The shorter term 'phage is commonly used for bacteriophage and will so be used here.



✓ **Animal Viruses.** In 1898, the first-known viral disease of lower animals (foot-and-mouth disease of cattle) was discovered by Loeffler and Frosch, while in 1900 Walter Reed, et al. discovered the virus of yellow fever, first known viral disease of man. Today many viral agents of disease of plants, insects, animals and man are well known.

#### GENERAL PROPERTIES

✓ With few exceptions viruses are characterized by: (1) size so minute as to make bacteria seem enormous by comparison; (2) inability to propagate outside of *living* cells (i.e., unlike bacteria, yeasts or molds they are *obligate parasites*). As a result of their minute size they cannot be seen with ordinary microscopes. They are, however, visible by means of electron microscopes. Their minute size also permits them to pass through clay, paper, asbestos or porcelain filters which entirely withhold bacterial cells. Because they are obligate parasites no saprophytic viruses are known but only those which produce recognizable disease.

✓ One of the most distinctive differential properties of all viruses is that of *host specificity*. Most viruses under *natural* conditions can infect only one single species or genus of animal or plant, or a very *closely similar* species; for example, polio virus infects monkeys and men but not cats. Tobacco mosaic virus may be made to infect the related tomato or potato plants but it does not infect any mammal, and mammalian virus does not infect any plant. Some insect-borne viruses appear to infect the transmitting insect and the plant or animal host. But these relationships are highly specific and restricted.

✓ On this basis we may divide all viruses into four large groups: (1) plant viruses; (2) mammalian viruses; (3) insect (or invertebrate) viruses; (4) bacterial viruses (called bacteriophage).

#### METHODS OF VIROLOGY

**Microscopy of Viruses.** The best microscopes with glass lenses and depending on visible light ("ordinary" microscopes\*) magnify at best not much over 1000 to 1500 diameters. This is far below the range of visibility of most viruses. By the use of electron microscopes (Chapter 9) magnifications up to 25,000 diameters are possible. Electronic images (which we call *electronographs* or electron micrographs) can be enlarged up to 100,000 as photographs. Photographs of electronographs of several viruses are shown in this chapter. Some idea of the range in size and form of viruses may be gained from a study of Figs. 5-1 to 5-10.

Some of the very largest mammalian viruses: smallpox, psittacosis ("parrot fever" or ornithosis), lymphogranuloma venereum, are large enough to be just visible with ordinary microscopes. They appear in tissue fluids as minute, refractile granules, often called *elementary bodies*. There is controversy as to whether these granules are actually the virus particles themselves or minute globules of tissue protein to which the viruses are attached by absorption, or clumps of virus particles. In dealing with mammalian viruses, even the largest

\* The common medical laboratory microscope (oil immersion) represents this type of instrument.

ones, this difficulty of confusion between tissue elements and actual virus always arises. However, there is reason to think that, in several instances, mammalian viruses have been electronographed in a fairly pure state.

Many of the plant viruses can be purified by precipitation and recrystallization. Their purity is well established and they lend themselves well to exact physical and chemical investigations. For example, tobacco mosaic virus, one of the most widely studied plant viruses, occurs as needle-shaped *liquid crystals* (the paracrystalline state). The crystals are doubly refractive, and possess positive electrical birefringence. The dimensions, as determined by calculation from sedimentation rates, specific viscosity and other physical data, are about 15  $m\mu$  by 120 to 280  $m\mu$ . Electron microscope findings give approximately the same figures. X-ray analysis yields information on molecular arrangements, etc.

**Chemistry and Physics of Viruses.** Chemically, the tobacco mosaic virus appears to consist of a central core of *nucleic acid* surrounded by a protective coating of *protein*. The two are probably loosely combined as *nucleoprotein*. Neither lipids nor carbohydrates, so common in all cellular organisms, are present.

Nothing resembling nucleus, cytoplasm or chromosomes is known though there certainly are definite and stable hereditary mechanisms. The virus structure appears to be non-cellular as we at present define a cell.

Egg Albumin Molecule	$9 \times 3$
Poliomyelitis Virus	12
Fowl Plague Virus	90
Vaccinia Virus	225
Psittacosis Virus	400
Rickettsiae of Typhus	
$300 \times 800$	
Micrococcus Pyogenes	
900	

Escherichia Coli  
 $7000 \times 1800$

Human Red Blood Corpuscle  
8000

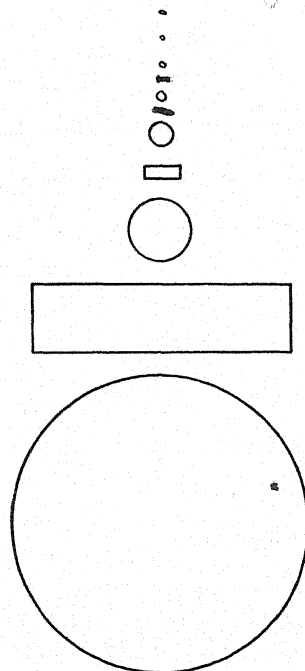


Fig. 5-1. Comparative sizes and forms of some microorganisms. The diagram gives only an approximate idea and is intended merely to suggest dimensional relationships. The dimensions are stated in millimicrons ( $m\mu = 0.001$  micron).

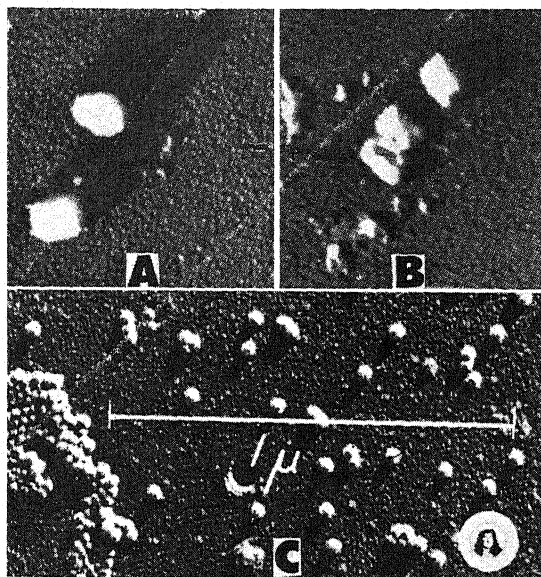


Fig. 5-2. Electronographs of representative animal viruses. *A* and *B* show the cuboidal form and relatively large size of one of the "large" viruses (vaccinia or smallpox-vaccine virus). The basic magnification was 24,800. *C* shows type I poliovirus. Note the smaller size (in spite of magnification of 73,500) and different form. Each separate poliovirus particle is about  $33\text{ m}\mu$  in diameter; those in clumps about  $25\text{ m}\mu$ . The particles may be spherical or polyhedral in form. (*A* and *B* courtesy of Drs. F. P. O. Nagler and G. Rake, in *J. Bact.*, vol. 55. *C*, from the collection of the Society of American Bacteriologists, Parke Davis and Co., Detroit.) (The small portrait of Antonij van Leeuwenhoek is in the emblem of the Society of American Bacteriologists.)

† A good deal has been learned about animal viruses, without purifying them, by subjecting them *in vitro*\* to various drugs, disinfectants, heat, radiations, etc., and then testing them *in vivo*† to see if they have been inactivated or have undergone mutation (heritable change) and the like. Even though they may not be alive (or infective) they may, as Salk found concerning polio virus, stimulate immunity in animals and serve as vaccines. †

**Viruses and Nucleoproteins.** It is of especial importance to note the presence of nucleic acids in viruses. In all known forms of reproduction, whether sexual or asexual, from man to bacterium, genetic material consists largely of nucleic acids, probably loosely combined with protein as nucleoprotein. ✓ It is therefore reasonable to assume that the nucleic acids of viruses constitute their genetic principle. (A diagram of the chemical makeup of nucleoproteins is shown in Figure 5-5.) Thus, tobacco mosaic virus, which in this respect exemplifies all other plant viruses, consists wholly (or almost wholly) of genetic material. This is of especial importance when we note that the *principal*, if not the *only*, physiological activity of viruses is *self-duplica-*

\* *In vitro* is derived from the Latin word, *vitrum*, for glass. It is commonly used to indicate experiments in the laboratory, in test tubes, not involving injection into experimental animals.

† *In vivo* indicates the experimental use of live animals.

tion. With few exceptions viruses appear to have no other physiological function and no metabolism. In short, most viruses behave very much as though they are (living?) genetic material. )

Animal viruses which have been studied are like sperm cells, in that they appear to contain a little carbohydrate and perhaps some lipid (fat-like) and other substances.\* But they also, like plant viruses, consist largely, if not entirely, of nucleoproteins (genetic material).†

SITE OF NUCLEIC ACIDS IN VIRUSES. Observations, suggestive of the structure of some mammalian viruses (smallpox, herpes, etc.) have been made

Fig. 5-3. A representative plant virus, the virus of tobacco mosaic disease. Note the crystal-like structure. Compare with bushy-stunt virus. The crystals are probably hexagonal in cross section. (Photo from the collection of the Society of American Bacteriologists, courtesy of Drs. R. C. Williams and R. W. G. Wyckoff, in *Science*, 1945, vol. 101.)

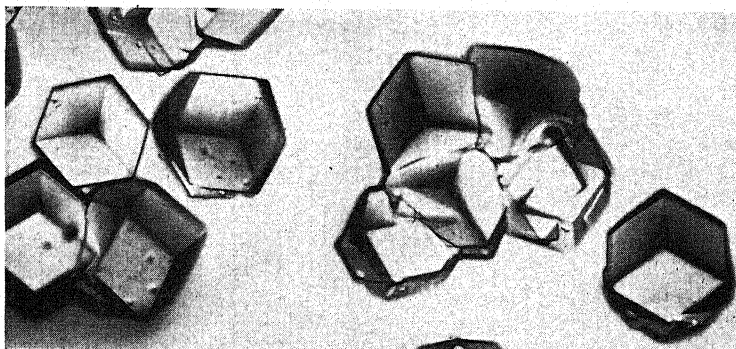
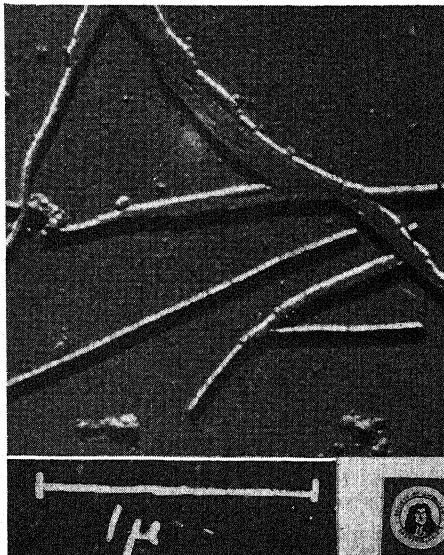


Fig. 5-4. Crystalline bushy stunt virus.  $\times 224$ . (Stanley, *J. Biol. Chem.*, vol. 135.)

\* As already indicated, the exact chemical composition of animal viruses cannot be too clearly stated because of the difficulty in separating them in a chemically pure state from the blood, tissues, etc., in which they occur.

† The sperm reproductive cells of all animals contain large amounts of nucleoproteins; also some carbohydrates and lipids.

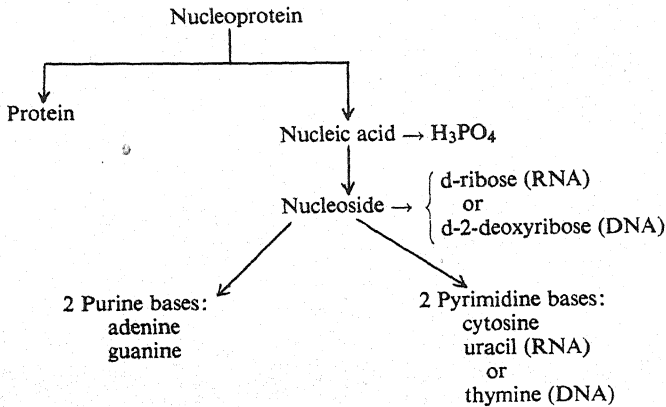


Fig. 5-5. Diagram of the structure of nucleoproteins as shown by hydrolysis. On being disintegrated by the action of hot acid or enzyme action, nucleoprotein first splits into a molecule of protein and one of nucleic acid. The latter, on further acid or enzyme treatment, yields a molecule of phosphoric acid, then a nucleoside which yields a molecule of carbohydrate: d-ribose if the nucleic acid is of the so-called "plant" or cytoplasmic type (ribonucleic acid or RNA), d-2-deoxyribose if the nucleic acid is of the so-called "animal" or nucleic type (deoxyribonucleic acid or DNA). The nucleoside further splits up into two purine bases, and two pyrimidine bases: cytosine and uracil if RNA; cytosine and thymine if DNA.

by preparing *very, very* thin sections of virus-infected cells, and staining by differential methods. Briefly, it is noted in such sections that each virus particle appears to consist of a central core of nucleic acid enclosed in a sort of membrane or capsule of protein. This type of structural form is also characteristic of viruses which infect insects (polyhedral insect viruses). Bacteriophages have a tadpole-like or sperm-like form with enlarged "head" and slender "tail." They also appear, pretty clearly, to consist of an inner core of DNA and an outer protective coat of protein. In each virus examined, there seems to be an inner structure which, we have good reason to believe, is the specific, active, self-replicating, nucleoprotein of the virus. This is protected by an outer coating of protein which appears to be inactive, non-specific, non-self-replicating (Figs. 5-6, 5-7, 5-8, 5-9). ✓

It is important to note that the nucleic acid of the nucleoproteins of plant viruses (with one main exception) is ribonucleic acid (RNA) while animal viruses contain either ribonucleic acid or deoxyribonucleic acid (DNA) or both. Bacteriophages contain DNA.

#### TISSUE CULTURES AND VIRUSES

Studies of viruses which involve animals or men (as was the case when Walter Reed used human volunteers to study the deadly yellow fever virus) are expensive and laborious. For many purposes it is now not necessary to use entire animals. All that animal viruses require for growth are *living animal tissue cells*.\* Now, we can easily induce animal tissue cells, for example monkey-kidney cells, to grow in test-tube cultures like bacteria. Then we can

\* Similarly, plant viruses require living plant cells, bacteriophages require living bacterial cells.

infect them with virus. By examining a few of the cells with microscopes every day we can observe the course of the infection. If the cells are damaged we say the virus is *cytopathogenic*.

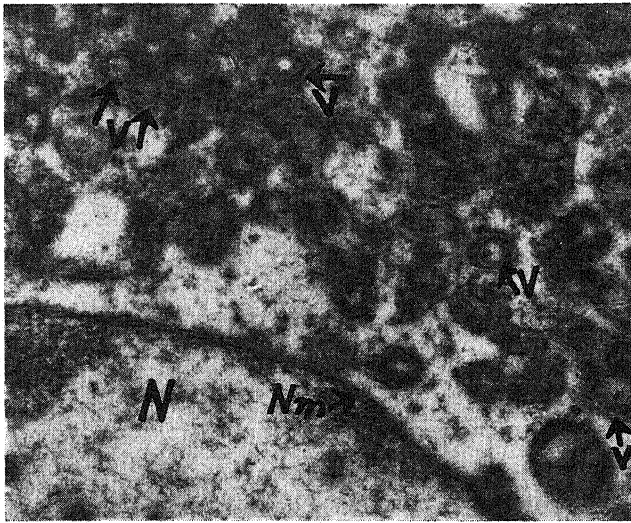


Fig. 5-6. Electronograph of a very thin cross section of a kidney cell infected with herpes B virus. In the cytoplasm are seen numerous rounded virus particles (*V*), with central "core" and outer "coat" or coats. (*N* = nucleus; *Nm* = nuclear membrane.) Similar particles are also found in cell nuclei as well as outside the cells. These particles in the cell constitute a typical intracellular inclusion. The original magnification was  $\times 36,000$ . (Photo courtesy of Drs. M. Reissig and J. L. Melnick; from *J. Exp. Med.*, 1955, vol. 101.)

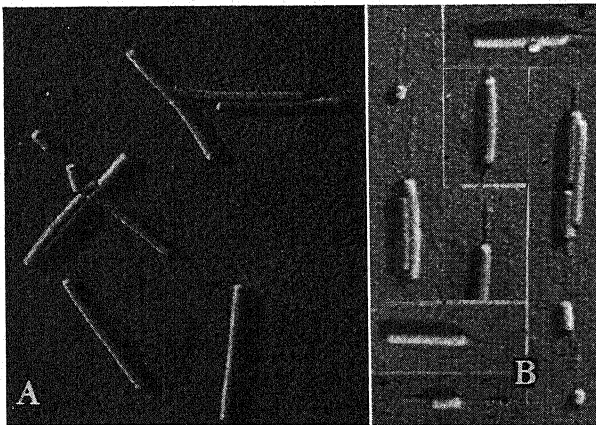


Fig. 5-7. Electronographs of tobacco mosaic virus (TMV). *A* shows the virus as usually seen ( $\times 78,000$ ). These rods appear to consist of hollow, round or hexagonal tubes of protein (94%) containing a thin core of ribonucleic acid (RNA) (6%). The RNA is the vital, specific, heredity-bearing part of the virus structure. It may exist as intertwining strands. *B* shows the TMV particles after treatment with a surface tension reducent which removed part of the protein coat from many of the rods, revealing the RNA core ( $\times 60,000$ ). Separated, neither coat nor core can infect. Recombined, they can infect. (Courtesy of Dr. R. G. Hart, in *Proc. Nat. Acad. Sci.*, 1955, vol. 41.)



Fig. 5-8. Electronographs of a representative insect virus (granulosis of the variegated cutworm). In *A* are seen complete virus particles, with a few bacteria to show comparative sizes and densities. In *B* the outer coats of the particles have been partly destroyed by washing with  $\text{Na}_2\text{CO}_3$  solution, revealing the inner core in some of the particles. In *C* the outer coat has been completely removed, leaving the rod-like inner cores. (From the collection of the Society of American Bacteriologists, courtesy of Drs. E. A. Steinhaus, K. M. Hughes and H. B. Wasser, in *J. Bact.*, vol. 57.)

#### MULTIPLICATION OF VIRUSES

Nothing resembling cell fission is known among viruses. Reproduction appears to be of a very special sort. It has been most thoroughly studied in bacteriophage because this virus is easily manipulated *in vitro*. We may use this as a model of viral multiplication though the process may differ considerably in some other viruses. Also, current ideas as described here vary somewhat and may alter as a result of future research. The basic ideas, though, are probably generally applicable.

Let us consider a cell of a common, harmless, bacterial species known as *Escherichia coli*. This bacterium is susceptible to infection by a certain, specific kind of 'phage ( $T_2$ ) of the group of 'phages designated as the T series.\*.

**Cell Receptors.** The exterior wall of the bacterial cell has a chemical structure which we may think of at present as a sort of molecular mosaic. This is made up of various arrangements of atomic groups or radicles ( $\text{R}\cdot\text{OH}$ ,  $\text{R}\cdot\text{COOH}$ ,  $\text{R}\cdot\text{NH}_2$ , etc.), with various electrical charges, polar groups, etc., associated with them. Let us suppose that a 'phage particle, infective for that bacterial cell, is carried close to the cell (probably by diffusion currents and brownian movement). Certain molecular arrangements and electrical charges at a particular site on the virus† correspond exactly‡ to a particular, specific, molecular pattern on the surface of the bacterium. This specific site on the cell is spoken of as a *receptor* for that 'phage. The 'phage tail attaches itself to the specific receptor of the cell (Fig. 5-10).

Within a few seconds a digestive (pepsin-like?) mechanism in the tail of the 'phage has made an opening in the bacterial cell wall. Through this the DNA

\* A series of these 'phages, which infect different varieties of *E. coli*, has been much used in experimental studies. They are usually designated as  $T_1$ ,  $T_2$ ,  $T_3$ , and so on.

† In bacteriophage this site is at the tip of the tail.

‡ Like a mirror image or like a key to a lock.



(genetic material) from inside the head of the 'phage is forced down through the tail, through the opening in the cell wall and so into the bacterial cell. The protein coating of the 'phage remains as an empty, inert shell on the outside of the cell as a "ghost" and eventually floats away. Its mission has been accomplished (Figs. 5-10 and 5-11).

**The Latent Period.** The nucleic acid of the 'phage disappears *as such*, inside the bacterial cell, for about twelve minutes. If the cells are ruptured experimentally during this time *no 'phage*, as such, can be demonstrated. This initial period, the duration of which is around twelve minutes, is quite constant for any given 'phage-bacterium system. It is the first half of what is called the *latent period* in viral infection of any cell. The latent period for influenza virus is from five to nine hours. It is more or less in other virus-cell systems.

**Formation of New 'Phage.** During the first twelve-minute period, in some unknown manner, with incredible rapidity, the 'phage enters the genetic government of the cell. 'Phage DNA takes over the synthetic mechanisms and causes them to synthesize, at first 'phage nucleic acid instead of bacterial material, and then 'phage protein coatings. At the end of the latent period, these portions begin to combine as 'phage particles. In about twelve minutes more the essential bacterial cell contents are wholly converted into 'phages.

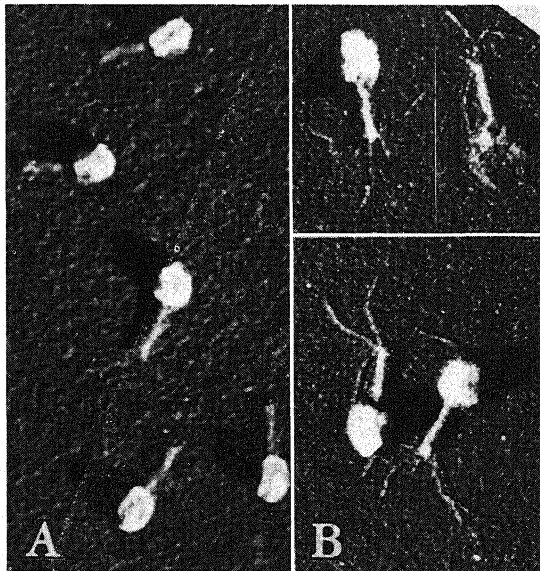


Fig. 5-9. Electronographs of bacteriophage ( $T_2$ ) of *Escherichia coli*. A shows the normal, tadpole-like, probably polygonal heads and tails in their usual form ( $\times 64,000$ ). B shows the 'phage after being frozen and thawed once. Note one ruptured head (upper right). The heads and tails often become separated (not shown here). The prominent caudal fibrils are believed to be normally twined closely around a protein core inside of the tail. Extruded, they appear to facilitate attachment of the virus particle to the bacterial surface and to remove the core from inside the tail. They are *not* fibers of RNA. ( $\times 68,000$ .) (Courtesy of Drs. R. C. Williams and D. Frazer, in *Virology*, 1956, vol. 2.)



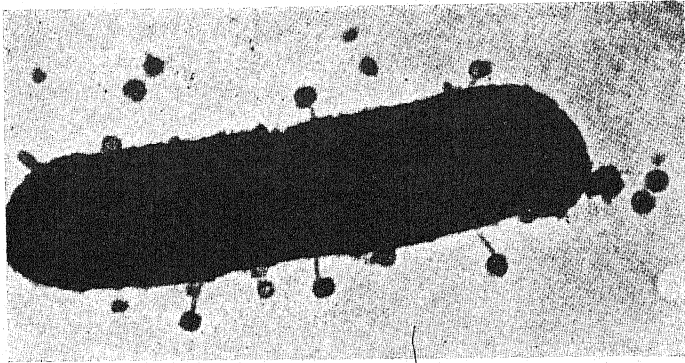


Fig. 5-10. Bacteriophage action on cells of *Escherichia coli*.  $\times 25,000$ . 'Phage particles adsorbed to a bacterium by their tails. Some heads are empty because the contents have passed through the tails into the bacterium. (Courtesy of T.F. Anderson from Cold Spring Harbor Symposium, vol. 18.)

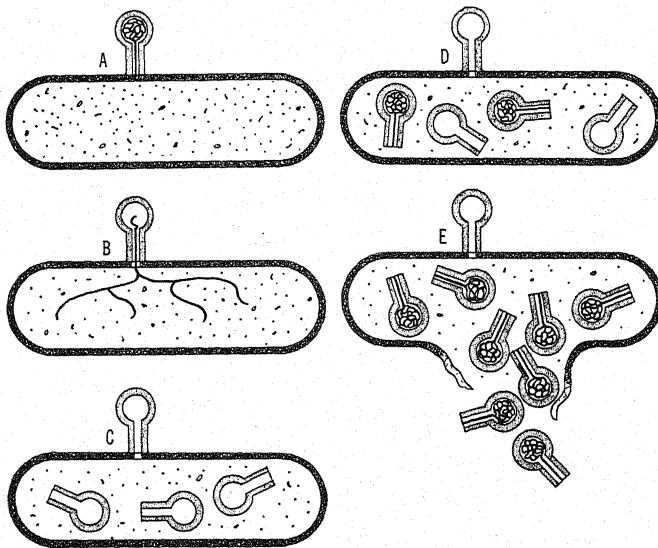


Fig. 5-11. Development of a virulent 'phage in a susceptible bacterium. At *A* the 'phage particle is seen attaching to the outer surface of the bacterium by its tail. In *B*, within a few seconds or minutes the bacterial cell wall has been penetrated and the DNA of the 'phage has entered the bacillus, leaving the inert protein coat on the outside of the cell. At *C*, about 12 minutes later, the 'phage as such is no longer demonstrable but protein coats are being synthesized. At *D*, about 12 minutes later, virulent 'phage particles are demonstrable, 'phage DNA having been synthesized by this time. At *E*, about 12 minutes later, the synthesis of 'phage is complete and the now "eviscerated" cell, an inert sac, ruptures liberating new 'phage particles; their number is characteristic of the burst size of the particular phage involved (in this case 8). The whole process, from *A* to *E*, occupies about 30 to 40 minutes.

**Cell Lysis.** Within about thirty minutes after attachment of the 'phage to the outside of the bacterial cell, the cell wall ruptures and the new 'phages are set free to begin the cycle anew. The bacterium is said to have undergone lysis.

The growth and metabolism of the 'phage have been cleverly investigated. As shown by radioisotope methods, the virus seems to derive some of its phosphorus and nitrogen from the medium in which the bacterium is growing, but about one fifth of each of these elements in the virus is derived from the bacterial cell itself.

**Burst Size.** The number of 'phage particles formed per cell in any given bacterium-'phage system is more or less constant. It may range around 20, around 50, 100, or 200 or more. This number is spoken of as the *burst size* for that 'phage-cell system. In the coli-T<sub>2</sub> 'phage system just described it is about 200.

**Resistance.** In preceding paragraphs we have described infection of a bacterial cell by a virulent 'phage, resulting in lysis of the cell. However, several other events may occur when a bacterial cell comes into contact with a 'phage to which it is usually susceptible.

First (continuing with our *E. coli*-T<sub>2</sub> system), the *E. coli* may, as a result of some mutation, have no receptors for T<sub>2</sub>. It is said to be a *resistant* strain. No 'phage T<sub>2</sub> can enter such a cell (though some other 'phage may do so).

Second, the cell may be too old. It is *important* to note that only multiplying bacterial cells, that is, cells in a stage when their *genetic material* is active and their outer coating presumably in a newly-formed condition, are susceptible to 'phage action or any virus action. Old, dormant bacteria are not affected.

**Virulent and Temperate 'Phage.** Third, the 'phage may contact the cell and the nucleic acid may enter, yet lysis not occur because the 'phage is not fully *virulent* for that cell; i.e., the virus is not able to (or for some other reason does not) produce lysis of the particular cell. Such a 'phage would be designated as a *temperate 'phage* or weak 'phage.

**REDUCTION.** Such a 'phage undergoes a curious change called *reduction*. It quietly enters the genetic structure of the cell and remains there, apparently as a gene.

The reduced virus often profoundly alters the heritable characteristics of the cell so that it seems like a new species. The 'phage is duplicated just like the bacterial DNA and is transmitted to the daughter cells when bacterial cell fission occurs. In all respects the temperate 'phage acts as though it were a normal part of the cell.

**Prophage.** It may remain there, latent for years and hundreds of millions of generations. In this state, it is spoken of as a *latent 'phage* or *symbiotic 'phage* or *prophage*. Unless some peculiar influence is brought to bear, as described under *Induction*, the presence of prophage in a species of bacteria may never be suspected. It is essentially a permanent part of the genetic mechanism (genome) of the cell.

**Lysogenicity and Induction.** A bacterial strain, in the cells of which prophage exists, is said to be *lysogenic*. If the prophage-carrying cells of that strain are subjected to certain influences such as ultraviolet irradiation, H<sub>2</sub>O<sub>2</sub>, excess vitamin C, and the like, the prophages are immediately stimulated into activity. They multiply as new, active, virulent 'phages, causing immediate lysis of their hosts, and liberating active, infective 'phages into the medium. The prophages are said to have been *induced*. Not all prophages are inducible. Inducibility may, like many other properties be altered by biological

mutation. Induction of prophages often occurs as a result of slight, unnoticed influences during ordinary laboratory manipulation. Thus, cultures of bacteria in which lysogenicity has never been suspected may suddenly undergo partial or complete lysis, revealing the presence of prophage in them. The culture is discovered to be lysogenic.

Whether all viruses multiply in exactly the same manner as 'phage is not entirely clear but available evidence indicates that they behave in a similar or analogous manner.

Like 'phage, they all behave like transmissible mutagenic agents; all\* have only a reproductive physiology; all are wholly dependent on other living cells for their life; all multiply only inside of those living cells, producing death and disintegration or mutation, directing the synthetic mechanisms of the infected cell to the making of virus substance. Animal viruses, like 'phages, show a latent period, varying from minutes to hours in various systems.

**Provirus.** Phenomena among animal viruses paralleling lysogenicity are exemplified by the virus of herpes simplex (fever blister). Nearly everyone has suffered the indignities inflicted by this virus. Persons who have had one attack apparently carry the virus in their tissue cells in a latent, prophage-like or *provirus* state. The provirus appears to be *induced* readily enough by such influences as sunburn (ultraviolet irradiation), increase in body temperature due to other infection (fever)—hence, “fever blisters.” There are other viruses which appear to exist as inducible proviruses.

**Resistance of Viruses.** In general, viruses are killed in a few minutes by temperatures like that of pasteurization†; some even as low as 56° C. There is at least one important exception: the virus of homologous serum jaundice (see Chapter 45). This stands boiling for some minutes. They are highly resistant to intense cold and will remain alive at -76° C for a year or longer. They are readily preserved by desiccation in vacuo after rapid freezing (freeze-drying or lyophilization). Ordinary strengths of disinfectants like phenol, cresol, formaldehyde and halogens quickly inactivate them. Surface-active agents like soap and some detergents appear to inactivate some viruses rather readily in vitro, not others. Ultraviolet light is rapidly destructive to all viruses.

**Viruses and Antibiotics.** In general, viral infections do not yield to treatment with antibiotics, sulfonamide drugs or other chemotherapeutic agents. We may postulate two reasons for this. First, since viruses multiply only *inside* the cell, chemotherapeutic drugs would not readily reach them. Second, all chemotherapeutic drugs appear to act by interfering with certain vital, specific, metabolic reactions in bacterial cells. Now, viruses appear to have no such vital, specific metabolic reactions. Thus, with two or three exceptions, there is nothing in viruses for any known chemotherapeutic agent to attack.

The two or three exceptions to this generalization are of interest. They are the large viruses: smallpox, psittacosis, lymphogranuloma venereum and others like them. These usually respond well to the so-called broad-spectrum antibiotics (Chapter 20). These viruses, as has been noted, are large enough to be just over the borderline of visibility with ordinary microscopes. Their structure, as seen with the electron microscope, is more complex than that of

\* With the possible exception of some of the large viruses.

† 62° C for 30 minutes.

other viruses, though not like what we ordinarily think of as a cell. Nevertheless, the fact that they yield to antibiotics suggests that they have some autonomous energy-yielding metabolism. This places them as a sort of intermediate form of life between the wholly dependent, degenerate, purely reproductive, small viruses and the wholly self-maintaining, cellular microorganisms like bacteria.

**Parasitic Status of Viruses.** Most bacteria can live independently of higher plants and animals. They thrive very well in the outer world. They are hardy and self-dependent, synthesizing their cell substance from simple foods from the outer environment. Species of bacteria which have become highly parasitic appear to have lost some of their rugged independence and their resistance to the outer world, through long generations of life in a sheltered environment such as the animal body. Here much of their food is synthesized for them. They tend to become dependent. Such bacteria must receive from their host in an already-synthesized state complex nutrient materials from the blood, or very complicated substances like vitamins, depending on how high a degree of parasitism (or dependence on host) they have evolved. They do not as a rule require, like viruses, to live *only inside* the host cells. Bacteria are mainly extracellular parasites.

Advancing a step further, we may imagine that certain bacteria have undergone such mutations in size and metabolism that they can actually enter the host cell and become an obligate intracellular parasite, capable of living *only there* at the expense of the proteins and other essential constituents of the cell, and losing more and more of their own synthetic and other physiological powers.\* As these powers are lost through mechanisms which induce cell variation, size diminishes until nothing remains but a bit of substance, probably nucleoprotein in nature, ultramicroscopic in size, able to pass through the finest filters, capable only of reproducing its kind through dominance over the genetic mechanism by inducing the host cell to alter its own substance to become the parasite; capable of life only if furnished with the cell-substances of some particular animal or plant to which it has become adapted. It would, "as it were, live a borrowed life, truly the supreme summit of parasitism."† Such a parasite would possess, as characteristic of itself, only the nucleoprotein transmitting the specific properties of the virus. It would resemble the gene (or plasmagene?) of the geneticists, independent and incarnate!

‡ So viruses may be supposed to have originated through the development of parasitism to its ultimate perfection; an evolutionary process of a highly successful sort if we regard first-rate parasites as the goal of Nature; an involutionary or degenerative trend if we regard the independent, self-supporting creature as the universal ideal. The latter, carried to its logical, non-parasitic extreme could be only of the completely autotrophic‡ type.

\* Indeed, just such visible but extremely minute, intracellular, bacterium- and virus-like parasites are well known and constitute an important group of organisms known as the rickettsiae which are discussed in Chapter 7.

† Laidlaw, "Virus Diseases and Viruses," 1939. By permission of The Macmillan Company.

‡ Capable of living on exclusively inorganic matter. Many such organisms are known. They are common in the soil.

**Classification of Viruses.** Classification of microorganisms is in a constant state of change and uncertainty. Classification of viruses is still in an embryonic, or at most early fetal, stage.

The International Committee on Bacteriological Nomenclature appointed a Subcommittee to deal with classification and nomenclature of viruses. There are three subcommittees: one for animal viruses, one for plant viruses (like tobacco mosaic) and one for bacterial viruses (bacteriophage). Work on all three projects is incomplete so that extensive discussion of classification of viruses, from the standpoint of proper nomenclature and taxonomy is not warranted here. In the 1948 edition of "Bergey's Manual," the viruses are included as the order *Virales*, divided into three sub-orders named Phagineae (the bacterial virus or bacteriophage); Phytophagineae, the viruses, like tobacco mosaic, causing plant diseases; Zoophagineae, the viruses, like yellow fever, causing diseases of man and animals. This classification, while having the advantage that nomenclature is at least documented, has not been generally accepted. The International Subcommittee on animal viruses tentatively adopted 8 criteria for classification:

1. Morphology and method of reproduction. (These might well be separated).
2. Chemical composition and physical properties.
3. Immunological properties.
4. Susceptibility to physical and chemical agents.
5. Natural methods of transmission.
6. Host, cell and tissue tropisms.
7. Pathology.
8. Symptomatology.

Interim names have been given some viruses, such as *Poliovirus hominis* (Type I, II, III). One group, called *Myxovirus*, includes influenza-like viruses (Types A and B). But so little is known for certain about the criteria used that final classification of all viruses awaits much more research.

A convenient, provisional "classification" of the viruses of animal disease may be based on the types of tissues or organs principally affected. Another sort of classification could be based on mode of transmission and another on type of disease caused. Such a list may be constructed as shown in Table 4.

Any such classifications, while convenient for purposes of discussion, are arbitrary and incomplete and cannot take into consideration the fact that viruses often become modified and cause atypical or entirely different sorts of disease. Further, the tissue affinities of some viruses can change completely. Finally, one is at a loss to place some viruses, such as that of mumps and of fowl plague in the table. Further, should the large viruses, which may be more closely related to rickettsiae, be included at all?

**Mutual Interference by Viruses.** It is clear that, if the specific receptors of cells are pre-empted by one virus, another having an affinity for the same receptors is excluded and cannot infect. This is mutual interference. The second virus could infect were it not restricted to certain receptors for its entry. For example, once certain plants become infected with the virus of tobacco mosaic they cannot be infected with a closely related variant of that virus. If monkeys are infected with a neurotropic variant of yellow fever virus,

they are immediately resistant to the normal yellow fever virus. If rabbits are infected with fibroma virus they are immediately wholly resistant to myxoma virus. In simultaneous inoculations with two viruses, especially if closely related, one pre-empts or infects; the other fails. Even inactive (irradiated) virus of influenza will preempt the susceptible cell receptors and prevent infection by live influenza virus. Viruses of canine distemper modified by passage

**Table 4.** *A Crude Classification of Some Animal Viruses (Zoophagineae).*

GROUP	TISSUE PRINCIPALLY AND VISIBLY AFFECTED*	TYPES OF DISEASE CAUSED	MODE OF TRANSMISSION*
Dermotropic	Skin; mucous membranes of nose and mouth	Various pox-like diseases (smallpox, fowl pox), herpes, warts, measles	Close contact; probably sputum, fomites
Neurotropic	Nervous tissues	Poliomyelitis Rabies Various encephalitides	Feces; sputum Bites of animals Mosquitos and other arthropods
Pneumotropic	Respiratory tract	Influenza, "colds," etc. Psittacosis Pneumonitis	Nasal and oral discharges
Viscerotropic	Various internal organs	Yellow fever Dengue Louping ill Rift Valley fever	Mosquitoes Mosquitoes Ticks Mosquitoes
Neoplastic	Various	Fowl sarcoma Fibroma of rabbits Myxoma of rabbits Fowl leukemia	Unknown Unknown Dust, fur, contact Unknown
Enteric	Gastrointestinal tract	Epidemic diarrhea, nausea and vomiting (?) Polio-like diseases	Probably by hands, foods and objects contaminated with feces

\* Under natural conditions.

through ferrets, and of low virulence for dogs and foxes, will eliminate fully-virulent distemper virus, not only if injected first, but even if given several days after the virulent virus.

The value of these observations in relation to the prevention and cure of viral infections is obvious. It may be, for example, that the true effect of Pasteur treatment for rabies lies less in development of antibodies than in blocking susceptible cells with a dead or attenuated virus so that the active virus from the bite of a rabid animal cannot take hold.

## REFERENCES

- Andrewes, C. H., Bang, F. B., and Burnet, F. M.: A short description of the Myxovirus group (influenza and related viruses). *Virology*, 1955, 1:176-184.
- Anonymous: Viruses. In "Research Reviews," J.A.M.A., 1956, 162:1583.
- Beard, J. W.: Physical and chemical characteristics of viruses. *Ann. Rev. Microbiol.*, 1951, 5:265.
- Brookhaven National Laboratory Symposium on Abnormal and Pathological Plant Growth. 1954. Office of Technical Services, U. S. Department of Commerce, Washington 25, D. C.
- Buddingh, G. J.: Chick embryo techniques. In Rivers', T. M., *Viral and Rickettsial Infections of Man*. 2nd ed. J. B. Lippincott Co., Philadelphia, 1952.
- Burnet, F. M.: *Viruses and Man*. Penguin Books, London, England, 1953.
- Burnet, F. M.: Principles of Animal Virology. Academic Press, Inc., New York, 1955.
- Burnet, Sir MacFarlane, and others: Virus and rickettsial classification and nomenclature. *Ann. New York Acad. Sci.*, 1952-1953, 56:381.
- Cox, H. R.: Growth of viruses and rickettsiae in the developing chick embryo. *Ann. New York Acad. Sci.*, 1952, 55:236.
- Dalldorf, G.: Introduction to Virology. Charles C Thomas, Springfield, Ill., 1955.
- Dalldorf, G.: The Coxsackie Viruses. *Ann. Rev. Microbiol.*, 1955, 9:277.
- Eagle, H., Habel, K., Rowe, W. P., and Huebner, R. J.: Viral susceptibility of a human carcinoma cell (Strain KB). *Proc. Soc. Exp. Biol. and Med.*, 1956, 91:361.
- Enders, J. F.: Cytopathology of virus infections. *Ann. Rev. Microbiol.*, 1954, 8:473.
- Evans, E. A., Jr.: Bacterial virus (with particular reference to the synthesis of). *Ann. Rev. Microbiol.*, 1954, 8:237.
- Evans, E. A., Jr.: Bacteriophage as nucleoprotein. *Fed. Proc.*, 1956, 15:827.
- Fraenkel-Conrat, H.: Rebuilding a virus. *Sci. Am.*, 1956, 194:42.
- Fraenkel-Conrat, H., and Williams, R.: Reconstitution of tobacco mosaic virus from its inactive protein and nucleic acid. *Proc. Nat. Acad. Sci.*, 1955, October. Cited from *Science*, 1955, 122:1880.
- Green, R. G.: Zoologic and histologic modification of the distemper virus by ferret passage. *Am. J. Hyg.*, 1945, 65:7.
- Harding, C. V., Harding, D., McLimans, W. F., and Rake, G.: Cytological changes accompanying the growth of poliomyelitis virus in cells of human origin (Strain HeLa). *Virology*, 1956, 2:109.
- Karzon, D. T., Barron, A. L., and Cohen, S.: Isolation of ECHO virus type 6 during outbreak of seasonal aseptic meningitis. *J.A.M.A.*, 1956, 162:1298.
- Lennette, E. H.: Interference between animal viruses. *Ann. Rev. Microbiol.*, 1951, 5:277.
- Lennette, E. H.: Symposium on newer knowledge of viral and rickettsial diseases. *Am. J. Trop. Med. and Hyg.*, 1956, 5:419.
- Lwoff, A.: Lysogeny. *Bact. Rev.*, 1953, 17:269.
- Lwoff, A.: The life cycle of a virus. *Sci. Am.*, 1954, 190:34.
- Melnick, J. L.: Viruses within cells. *Sci. Am.*, 1953, 189:39.
- Morgan, C., Ellison, S. A., Rose, H. M., and Moore, H. H.: Internal structure of virus particles. *Nature*, 1954, 173:208.
- Moore, A. E. Effects of viruses on tumors. *Ann. Rev. Microbiol.*, 1954, 8:393.
- Pearson, H. E.: Biochemical aspects of viral growth. *Ann. Rev. Microbiol.*, 1953, 7:179.
- Pollard, E. C.: The physics of viruses. *Sci. Am.*, 1954, 191:63.
- Price, W. H.: Bacterial viruses. *Ann. Rev. Microbiol.*, 1952, 6:333.
- Rhodes, A. J., and van Rooyen, C. F.: *Textbook of Virology*. Williams & Wilkins Co., Baltimore, Md., 1954.
- Rivers, T. M., Editor: *Viral and Rickettsial Infections of Man*. J. B. Lippincott Co., Philadelphia, 1952.
- Schlesinger, R. W.: Developmental stages of viruses. *Ann. Rev. Microbiol.*, 1953, 7:83.
- Smith, K. M., and Lauffer, M. A., Editors: *Advances in Virus Research*. Academic Press, Inc., New York, 1953, 1954, 1955.
- Society for General Microbiology Symposium No. 2: The Nature of Virus Multiplication. Fildes, Sir Paul, and van Heyningen, W. E., Editors, 1952, Cambridge University Press.
- Stanley, W. M.: Virus composition and structure—25 years ago and now. *Fed. Proc.*, 1956, 15:812.

- Stent, G. S.: The multiplication of bacterial viruses. *Sci. Am.*, 1955, 188:36.
- Various Authors. Conference of the similarities and dissimilarities between viruses attacking animals, plants and bacteria. Pasadena, Calif., California Institute of Technology, 1950.
- von Magnus, H., Gear, J. H. S., and Paul, J. R.: A recent definition of poliomyelitis viruses. *Virology*, 1955, 1:185.
- Weiss, E.: The nature of the psittacosis-lymphogranuloma group of microorganisms. *Ann. Rev. Microbiol.*, 1955, 9:227.



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### 6. THE VIRUSES. B. BACTERIOPHAGE

BECAUSE OF the ease, speed and economy with which they may be cultivated in living bacterial cells, their relative resistance to long storage in refrigerators and to general laboratory manipulation, and their distinctive appearance and properties, the bacteriophages have been much used as experimental models of viruses in general. Their peculiarities have also been used for several practical purposes; for example, several 'phages are of considerable industrial importance. For these reasons there are described here some details concerning 'phages that were not given in the previous chapter on viruses.

Bacteriophage was first described by Twort (1915) and later (1917) by d'Herelle and its action is often called the Twort-d'Herelle phenomenon. Bacteriophage may be found in many situations in nature where bacteria are growing and are especially abundant in the intestine of man, animals and insects, sewage and soil.

**Isolation of 'phage.** If a small amount of sewage, feces or ground-up flies or roaches is emulsified with broth and passed through a filter fine enough to retain bacteria, the filtrate\* will often be found to contain this virus. The bacteria may also be removed by high-speed centrifugation or by differential heating. The 'phage may be demonstrated in the bacteria-free fluid by adding a drop or two to a very young, actively growing, slightly turbid, broth culture of appropriate bacteria, such as virulent dysentery or typhoid bacilli.

The culture will be found, after a few hours' incubation, to have become nearly or entirely clear and the live bacilli in it either reduced in numbers or entirely absent. If, now, this broth be rendered bacterium-free and a drop of it added to another young broth culture, this in turn will clear, and filtrates from it will induce the clearing phenomenon in other cultures. It is necessary to use *young* broth cultures because 'phage does not multiply in old cells or in any which are not actively multiplying; i.e., in any in which the *genetic mechanisms* are not active. The lytic† potency of the filtrates increases with

\* Fluid passing through the filter.

† Dissolving or digesting.

each transfer, the 'phage multiplying enormously at each transfer and accomplishing the clearing much more rapidly and completely than at first. In addition to the increased activity of the 'phage, it usually becomes more and more specific for the bacterium on which it grows and less active on other species.

If a loopful of the partly lysed (cleared) broth culture is smeared on an agar plate,\* any surviving bacteria that grow into colonies are often found to be distinct variants from the original. This may be the effect of *reduction* of 'phage in some of the cells. It has been postulated that new species may originate in this way. Of course, they also may have been present all the time as mutants in the culture, being merely revealed by the lysis of their fellows.

As pointed out in Chapter 5, 'phages (and probably other viruses) can act like *living mutagens* or migratory genes once they attach themselves to susceptible cells.

**Transduction.** In addition to entering the genetic mechanism of the bacterial cell, the 'phage apparently can acquire some portion of the bacterial genetic material and transfer it to another bacterium. For example, 'phage from a motile cell of a certain species can "infect" the cell of a closely related but non-motile species, causing the non-motile species to become a motile species! Biological Magic! The phenomenon of transfer of genetic material by 'phage is called *transduction*. It is discussed more fully in Chapter 15.

**Plaque Formation.** Multiplication of 'phage may also be made evident by smearing an agar plate with a drop of the young, active culture *before* putting in the 'phage, and then smearing another agar plate with the same culture a few minutes after the addition of the 'phage. The first plate will show profuse and evenly distributed normal growth in a smooth, gray sheet, unbroken except in the most thinly seeded areas where there are separate colonies. These will have smooth and regular edges. The second plate will show growth dotted here and there with "pinholes" or places where no visible growth has occurred. These "pinholes" are called 'phage *plaques*. Each may be regarded as a "colony" consisting of billions of particles of 'phage, which has destroyed the bacteria around it (Fig. 6-1). Isolated bacterial colonies on this plate may show crescentic irregularities in their margins, as though pieces had been eaten out of the edges. This is due to the lytic action of the 'phage in those colonies. The phenomenon of plaque formation is most easily demonstrated with 'phage but is not restricted to the bacterial virus. It has been shown to occur in an analogous manner with animal viruses multiplying in a layer of animal cells. For example, cells of a rat sarcoma† have been cultivated as a layer in a flask lined with plasma clot. The growing cells settled and formed a smooth, gray, unbroken sheet of growing cells on the bottom of the flask. This is wholly analogous to the sheet of bacterial growth on an agar plate as described above. Instead of 'phage, a virus capable of infecting the rat sarcoma cells was introduced. Plaques formed in the sheet of sarcoma cells exactly as in 'phage plaque formation. Similar plaque formation has been much used in studies of polio virus, using sheets of actively growing monkey-kidney cells

\* A nutrient jelly surface on which bacteria can grow.

† A neoplasm related to cancer.

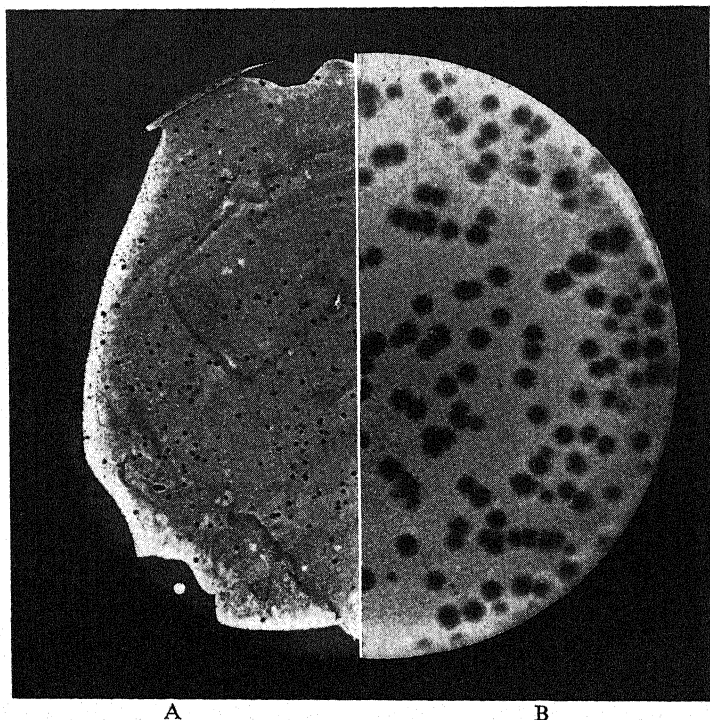


Fig. 6-1. Plaque formation by phage. The dark "holes" are plaques. *A* is a "small plaque" variety of 'phage. *B*, a large plaque variety of 'phage. Note the irregular edges of the plaques. Minute colonies of resistant bacteria are often found in the area of the plaque. 'Phages often undergo mutations which can be recognized as distinctive appearances in the size, form, margin, etc., of the plaques formed. (Courtesy of Society of American Bacteriologists.)

in cultures (in place of bacteria) and polio virus (in place of 'phage). The plaque technique has been applied to several other virus-cell systems.

**Enumeration of Bacteriophage.** It is obvious that the number of plaques formed will be related to the number of 'phage particles in the 'phage-bearing fluid. This suggests the possibility of actually counting the 'phage particles and expressing their number (per unit volume of fluid) as 'phage *titer*.<sup>\*</sup> This may be done by two methods. In one, graded quantities of the 'phage suspension are added to constant amounts (say 5 ml) of young broth cultures of the susceptible bacterium. After appropriate incubation, the highest dilution of 'phage suspension producing complete or perceptible clearing in the cultures is noted as the titer of 'phage.

The second method depends on counting the number of plaques produced on the surface of plates, inoculated as described above, with graded dilutions of the 'phage suspension. A similar procedure is used to enumerate virus particles in various other virus-cell systems; e.g., a polio-virus-monkey kidney-cell system.

<sup>\*</sup> Titer is from the French word *titre*, meaning a standard of value or measure. It is generally used to indicate concentration or amount per volume.

**Effect of Colloidal Matter on Bacteriophage.** When bacteriophage was first discovered, it was thought that disease could be cured with it. If, for example, persons with cholera could swallow cholera 'phage or have it injected into them, it was thought that the 'phage would destroy the cholera vibrios in the intestine and thus cure the disease. Similarly, it was thought that persons suffering from boils could have anti-*Micrococcus pyogenes* 'phage injected, or applied in compresses, with a resulting destruction of the micrococci and cure of the boils. It was soon found that, unfortunately, bacteriophage will not act well in the presence of blood, pus, fecal or any colloidal material. It is not generally used in therapy of infections.

**Varieties of Bacteriophage.** Bacteriophages active against certain kinds of bacteria have been found, but none against others.

'Phages active against the mold-like group of bacteria, Actinomycetales, are rare. However, an *actinophage* against *Streptomyces griseus*, the organism from which the antibiotic streptomycin is obtained, has been isolated. Seen by means of electronographs some of the particles of this virus appear to have two tails each. The 'phage attacks the *Streptomyces* growing in the industrial culture vats used for production of streptomycin and can be an expensive nuisance.

Another industrially important 'phage is one active against the bacteria (*Streptococcus lactis* and related species) which are used to cause souring of

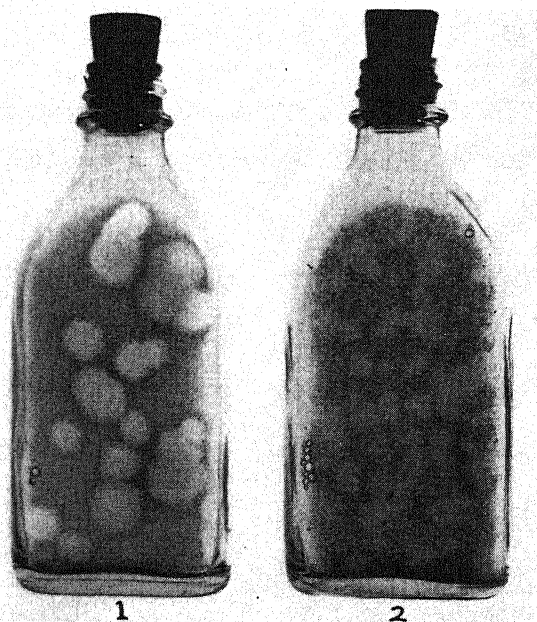


Fig. 6-2. Plaques made in monkey epithelial cells cultivated in bottles in fluid culture medium. The cells were mixed with a suspension of virus particles and then held in place in a thin layer by adding agar which then solidified. Each virus particle attacked the surrounding tissue cells, causing the formation of a plaque analogous to 'phage plaques. 1, polio-virus (type 3) plaques; 2, ECHO virus (type 6) plaques. (Photos courtesy of Drs. G. D. Hsiung and J. L. Melnick; from *Virology*, 1955, vol. 1.)

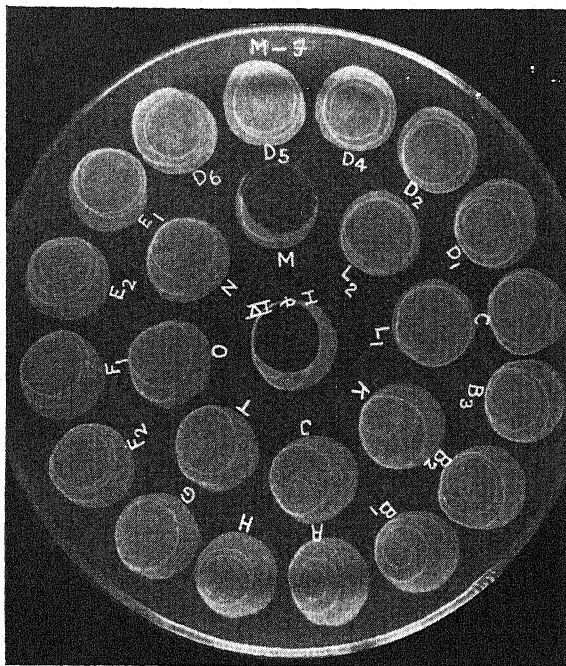


Fig. 6-3. Use of highly specific bacteriophages to type typhoid bacilli. Separate drops of a culture of *Salmonella typhi* of unknown type have each been mixed with a different type of *Salmonella typhi* bacteriophage (indicated by letters) on the plate. Lysis (circular dark area in the white growth) occurs only where 'phage type and bacillus type correspond; in this case, type M. The lysis in the center of the plate is a control test. Closely parallel processes are used in the 'phage typing of staphylococci, *Shigella*, and other bacteria. (Specimen prepared by Dr. Philip R. Edwards. Photo courtesy of U. S. Public Health Service, Communicable Disease Center, Atlanta, Ga.)

milk and cream for butter and cheese making, and to produce lactic acid in other dairy products like yoghurt and buttermilk. When such a 'phage (and they are widely distributed) gets into the creamery vats whole batches of valuable culture-soured dairy products are spoiled. The control of such 'phages is an important industrial problem. One method consists in selecting genetically-resistant strains of milk-souring streptococci.

Bacteriophages have been found in nature active against a wide variety of bacteria, including tubercle bacilli, diphtheria bacilli, certain very valuable bacteria (*Azotobacter*) of the soil (a serious problem to agriculture), spore-forming bacilli of the genus *Bacillus*, and numerous others.

There is said, by some students of the subject, to be only one bacteriophage and that, by suitable *adaptation* (i.e., continuous propagation in contact with the desired bacterial species), it can be made to attack selected, specific species of bacteria. There is no doubt that 'phages are readily capable of adaptation in very marked degree. Others explain this apparent adaptation as resulting from *selective growth* of one kind of 'phage (though perhaps initially present as only one mutant particle among billions of other particles).

When placed in contact with bacteria suitable to only *that one* mutant particle all others are killed while that one multiplies. This is a matter still in dispute.

**"Typing" of Bacteria with Bacteriophage.** The process of adaptation (or selective cultivation) of bacteriophage may be carried to a degree where a given strain of 'phage becomes so selective with respect to a single species of bacterium, or even on a certain type or subdivision of that species, that it will not act on any other. Thus, it will distinguish subdivisions or types among apparently identical strains which are indistinguishable by any other means. For example by propagating a given 'phage on a certain selected V\* strain† of *Salmonella typhi*‡ (typhoid bacillus), the 'phage becomes so specific for that particular strain that, *when appropriately diluted*§ it will not act on any other strain of the same species. The first highly specific typhoid 'phage of this sort was designated as "typhoid 'phage A" and the corresponding susceptible strain of typhoid bacilli as "'phage-type A of *S. typhi*." By a like process several other 'phage types of *S. typhi* were discovered, and were designated by letters A, B, C, D, E, etc. The use of such a system of 'phages to detect an M type of *S. typhi* is seen in Figure 6-3.

Similar bacteriophage typing systems have been developed for several other species of bacteria, notably *Micrococcus* and dysentery bacilli.

#### REFERENCES

- Bennett, F. W., and Nelson, F. E.: Action of aerosols of certain virucidal agents on lactic streptococcus bacteriophage. *J. Dairy Sci.*, 1954, 37:840.  
Brown, E. R., and Cherry, W. B.: Specific identification of *Bacillus anthracis* by means of a variant bacteriophage. *J. Inf. Dis.*, 1955, 96:34.  
Brown, E. R., Cherry, W. B., Moody, M. D., and Gordon, M. A.: The induction of motility in *Bacillus anthracis* by means of bacteriophage lysates. *J. Bact.*, 1955, 69:590.  
Cherry, W. B., Davis, B. R., Edwards, P. R., and Hogan, R. B.: A simple procedure for the identification of the genus *Salmonella* by means of a specific bacteriophage. *J. Lab. and Clin. Med.*, 1954, 44:51.  
Cooper, P. D.: A method for producing plaques in agar suspensions of animal cells. *Virology*, 1955, 1:397.  
d'Herelle, F.: *The Bacteriophage and Its Behavior*. Williams & Wilkins Co., Baltimore, Md., 1926.  
Dulbecco, R., and Vogt, M.: Biological properties of poliomyelitis viruses as studied by the plaque technic. *Ann. New York Acad. Sci.*, 1955, 61:790.  
Evans, Alice C.: Inactivation of anti-streptococcus bacteriophage by animal fluids. *Pub. Health Rep.*, 1933, 48:411.  
Felix, A.: 'Phage typing of *Salmonella typhimurium*: its place in epidemiological and epizootiological investigations. *J. Gen. Micro.*, 1956, 14:208.  
Fogh, J., and Lund, R. O.: Plaque formation of poliomyelitis viruses on human amnion cell cultures. *Proc. Soc. Exp. Biol. & Med.*, 1955, 90:80.

\* Varieties having a certain form of colony called "smooth." There are four forms of V 'phage: I, II, III and IV. 'Phages I and IV attack any V-strain of *S. typhi*. The highly specific 'phages are developed from II. Rough colony forms of *S. typhi* are spoken of in this system as W forms. They are not susceptible to 'phage and cannot be typed. In Figure 6-3 the central lysis is by 'phages I and IV to show that a V form of *S. typhi* is being dealt with.

† Any designated specimen or culture, or progeny of same.

‡ This term, while not official in the 6th edition of Bergey's Manual, is proving more acceptable and will probably be made official in the next edition of the manual.

§ If not diluted, the selective specificity is masked by an overwhelming action on all V forms of typhoid bacilli.

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- Fusillo, M. H., Rierig, R. N., Metzger, J. F., and Ernst, K. F.: Phage typing antibiotic-resistant staphylococci. *Am. J. Pub. Health*, 1954, *44*:317.
- Granoff, A.: Plaque formation with influenza strains. *Virology*, 1955, *1*:252.
- Hsiung, G. D., and Melnick, J. L.: Plaque formation with poliomyelitis, Cocksackie, and Orphan (ECHO) viruses in bottle cultures of monkey epithelial cells. *Virology*, 1955, *1*:533.
- McAllister, R. M., and Coriell, L. L.: Cultivation of human epithelial cells in tissue culture. *Proc. Soc. Exp. Biol. and Med.*, 1956, *91*:389.
- Price, W. H.: Bacterial viruses. *Ann. Rev. Microbiol.*, 1952, *6*:333.
- Takemori, N., Nakano, M., and Hemmi, M.: Plaque formation with Rift Valley fever virus. *Virology*, 1955, *1*:250.
- Wasserman, M. M., and Saphra, I.: The use of bacteriophage in typing *Salmonella* cultures. *J. Bact.*, 1955, *69*:97.
- Whitehead, H. R.: Bacteriophage in cheese manufacture. *Bact. Rev.*, 1953, *17*:109.

## The Microscopic World

### 7. THE RICKETTSIAE

#### DISCOVERY

HOWARD TAYLOR RICKETTS, an American medical scientist, while studying Rocky Mountain spotted fever in 1909, described as the causative agents of that disease a group of microorganisms which differed from any previously known. A year later he discovered similar organisms as the cause of typhus\* fever while working in Mexico. During the latter studies he contracted the disease and died.

In 1916, H. da Rocha-Lima, a Brazilian scientist, made further observations of the organisms described by Ricketts and named them *Rickettsia* in honor of their discoverer. He also gave the name of *proWazekii* to the rickettsiae associated with typhus fever, in honor of another scientist, Stanislaus von Prowazek, of Hamburg, who had lost his life in the study of that disease. The causative agent of louse-borne typhus fever is, therefore, called *Rickettsia proWazekii*. It is the type species of the genus *Rickettsia*.

#### CHARACTERISTICS OF RICKETTSIAE

The rickettsiae have some properties of viruses and also some properties of bacteria. In size they are intermediate between the large viruses and the bacteria. They are clearly not yeasts, molds, protozoa, PPLO or algae. Most probably they are neither viruses nor bacteria, but an intermediate group. (See Table 1.)

**Morphology.** The organisms are very tiny, having diameters of about 0.3  $\mu$  and lengths seldom exceeding 2  $\mu$  and often less than this. However, they are larger than the large viruses and can readily be seen with ordinary microscopes. They appear to multiply by fission like other cellular organisms, but this is not certain. They are variously shaped, like minute bacilli, cocci, or

\* Do not confuse typhus fever, a blood and tissue disease due to rickettsiae, with typhoid fever, an intestinal infection due to a bacterium, *Salmonella typhi*. The name of typhoid fever was given it by a French physician contemporary with Marie Antoinette (Dr. Pierre Louis) because the stuporous symptoms of typhoid in some ways resemble those of typhus. The word typhus means stuporous or drowsy.



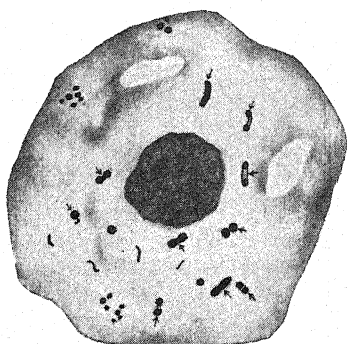


Fig. 7-1. Epithelial cell showing rickettsiae in the cytoplasm. Typical rickettsiae are indicated by arrows. (About  $\times 3000$ .)

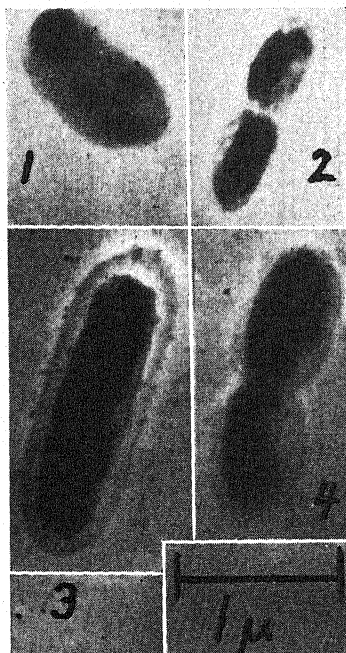


Fig. 7-2. Electronographs of various representative rickettsiae. Note the bacterium-like form and structure; with cell wall, cytoplasm with intracellular granules, and what appears to be binary fission. Note also the small size as compared with bacteria. 1, *R. mooseri*; 2, *R. (Coxiella) burneti*; 3, *R. rickettsii*; 4, *R. prowazekii*. From yolk-sac cultures. (From the collection of the Society of American Bacteriologists, courtesy of Drs. H. Plotz, J. E. Smadel, T. F. Anderson, and L. A. Chambers, in *J. Exp. Med.*, vol. 77.)

diplococci\* (Figs. 7-1 and 7-2). Sometimes relatively long filaments are formed. No spores† are produced. The organisms are not motile.

Unlike viruses, rickettsiae are not filtrable‡ and in this respect resemble bacteria.

Unlike bacteria, it is difficult to stain them with ordinary basic aniline dyes. They can, however, be colored with Giemsa's stain.§ The rod forms tend to stain more intensely at the tips, often giving short rods the appearance of a pair of diplococci.

\* When cocci occur in pairs, they are called diplo-cocci.

† Spores are dormant, seed-like bodies produced by some species of bacteria and higher fungi.

‡ With the single exception of *Rickettsia burneti*, the cause of Q fever.

§ A special stain used for blood and protozoa.

Photographic enlargements of electronographs, and specially designed light microscopes (phase microscopes, see Chapt. 9) indicate that rickettsiae have internal structures suggesting the existence of nuclei or nucleus-like mechanisms. They also appear to contain both ribonucleic acid and deoxyribonucleic acid, components of cytoplasm and nuclei, respectively (Fig. 7-2).

**Growth.** In the matter of growth, rickettsiae resemble the viruses; i.e., they are not cultivable on non-living material. However, rickettsiae are easily cultivated in *live* chick embryos and in *living* tissue-cultures like those used to cultivate viruses (see Chapt. 13). They appear to grow best in the live cells lining the egg-yolk sac. Yolk-grown rickettsiae are widely used in procedures for the diagnosis of rickettsial diseases and for the preparation of rickettsial vaccines.

**Rickettsiae and Antibiotics.** Some rickettsiae have been shown to have a definite, autonomous, metabolic activity, although they have not been cultivated on lifeless media. This probably explains why they are markedly susceptible to antibiotics, since antibiotics, as already noted, act by interfering with certain essential metabolic processes of living cells. The large viruses, as previously indicated, are also susceptible to antibiotics, and appear to have some independent metabolic functions and some structures like rickettsiae. Some authorities would classify the large viruses with the rickettsiae.

**Table 5.** *Some Typical Rickettsial Diseases.*

DISEASE	RICKETTSIAE	ARTHROPOD VECTOR	MAMMALIAN HOST
Rocky Mountain spotted fever	<i>R. rickettsii</i>	Dog tick ( <i>Dermacentor variabilis</i> ); Rabbit tick ( <i>Dermacentor andersoni</i> )	Rabbits, dogs, sheep; (Man)*
Murine (endemic) typhus	<i>R. mooseri</i>	Rat flea ( <i>Xenopsylla cheopis</i> )	House, barn and sewer rats; (Man)*
Classical (epidemic) typhus	<i>R. prowazekii</i>	Body louse ( <i>Pediculus corporis</i> )	Man
Tsutsugamushi (Japanese, or Oriental swamp or river fever)	<i>R. orientalis</i>	Harvest mite ( <i>Trombicula akamushi</i> )	Field mice and other small rodents; (Man)*
Rickettsialpox	<i>R. akari</i>	Mouse mite ( <i>Allodermanyssus sanguineus</i> )	House mouse; (Man)*
Brill's disease (recrudescence typhus)†	<i>R. prowazekii</i>	Body louse	Man
Q fever	<i>R. burneti</i>	Ticks, milk, dust	Cattle; (Man)*

\* The human mammalian host is placed in parentheses (except in classical typhus) because he is not the usual or natural host but usually a chance or accidental victim of the vector insect.

† Represents reactivation of an old infection, the organisms having remained in the body in a quiescent state, sometimes for several decades.

**Habitat.** The rickettsiae are obligate, intracellular parasites, like viruses. They characteristically inhabit the cells lining the intestines and other tissues of insects, both bloodsucking and non-bloodsucking. Rickettsiae not pathogenic for man have been found in ticks, fleas, lice, bedbugs, spiders and mosquitoes. Human pathogenic species of rickettsiae primarily inhabit insects which bite man or animals or both. Many pathogenic rickettsiae are found only in the salivary glands of the insects, whence they may be transmitted to man. However, they sometimes occur in the intestinal contents of bloodsucking insects. They therefore appear in the feces. Transmission of rickettsiae to animal hosts may thus be obtained by rubbing or scratching the fecal deposits of insect vectors into the skin.

#### RICKETTSIAL DISEASES

Some of the principal diseases caused by rickettsiae are shown in Table 5. As seen in this table they are primarily diseases of lower animals. Some of the rickettsiae contain potent poisons (toxins).

#### REFERENCES

- Bell, E. J., and Philip, C. B.: The human rickettsioses. *Ann. Rev. Microbiol.*, 1952, 6:91.  
 Bovarnick, M. R.: Rickettsiae. *Sci. Am.*, 1955, 192:74.  
 Buddingh, G. J.: Chick embryo techniques. *In* Rivers, T. M., *Viral and Rickettsial Infections of Man*. 2nd ed. J. B. Lippincott Co., Philadelphia, 1952.  
 Cox, H. R.: Growth of viruses and rickettsiae in the developing chick embryo. *Ann. New York Acad. Sci.*, 1952, 55:236.  
 Hopps, H. E., et al.: Metabolic studies of rickettsiae. *J. Bact.*, 1956, 71:708.  
 Neva, F. A., and Snyder, J. C.: Studies on the toxicity of typhus rickettsiae. *J. Inf. Dis.*, 1955, 97:73.  
 Price, W. H., Johnson, J. W., Emerson, H., and Preston, C.: Rickettsial-interference phenomenon: a new protective mechanism. *Science*, 1954, 120:457.  
 Rivers, T. M., et al.: *Viral and Rickettsial Infections of Man*. 2nd ed. J. B. Lippincott Co., Philadelphia, 1952.  
 Smadel, J. E., et al.: Symposium on viral and rickettsial diseases. *Bact. Rev.*, 1950, 14:195.  
 Zinsser, H.: *Rats, Lice and History*. Little, Brown and Co., and The Atlantic Monthly, Boston, Mass., 1935.

## The Microscopic World

### 8. THE PLEUROPNEUMONIA AND PLEUROPNEUMONIA-LIKE ORGANISMS

#### PRINCIPAL GROUPS

**Pleuropneumonia Organisms.** In 1898 Nocard and Roux, French scientists, discovered, in pleural\* fluids from cattle with a disease called infectious pleuropneumonia, filtrable organisms of the same range of magnitude as the larger, rickettsia-like viruses, being just within the range of visibility of ordinary, light microscopes. However, the pleuropneumonia organisms differ from all viruses (as well as from rickettsia) in being cultivable on lifeless media (Table 6). On agar cultures minute, round granules, not unlike the elementary bodies of the large viruses, are seen in colonies. Long, mold-like but very thin filaments, and stellate structures are also formed by some species. Often they are spherical or ovoid. It is clear that an outstanding character of these organisms is *pleomorphism* (Figs. 8-1 and 8-2). Borrel (for whom the genus of *Borrelia* is named) called the species discovered by Nocard and Roux *Asterococcus mycoides*, meaning "rounded and stellate forms with radial, mold-like filaments." These organisms are commonly called *pleuropneumonia organisms*, which we may, for convenience in this discussion, abbreviate to PPO.

**Pleuropneumonia-like Organisms.** After the original description of the bovine pleuropneumonia organisms, similar species were found in various other animals: sheep, goats, dogs, rats, mice, human beings. They are associated with various pathologic conditions, especially rheumatic or arthritic diseases, infections of the mammary glands, respiratory tract and adjacent tissues, and inflammations of the genito-urinary system. In addition, one group of similar organisms was found growing as saprophytes in decaying organic matter. All of these organisms are now referred to as *pleuropneumonia-like organisms*, commonly called PPLO.

**L Forms.** A group of organisms resembling PPO and PPLO in several respects, and usually included with them, are often called *L bodies* or *L forms*. There appear to be three distinct types, L<sub>1</sub>, L<sub>3</sub> and L<sub>4</sub>.† The L bodies seem to

\* Surrounding the lungs.

† Some authors have called certain PPLO "L<sub>2</sub>," "L<sub>3</sub>," "L<sub>4</sub>," causing some confusion in the literature.

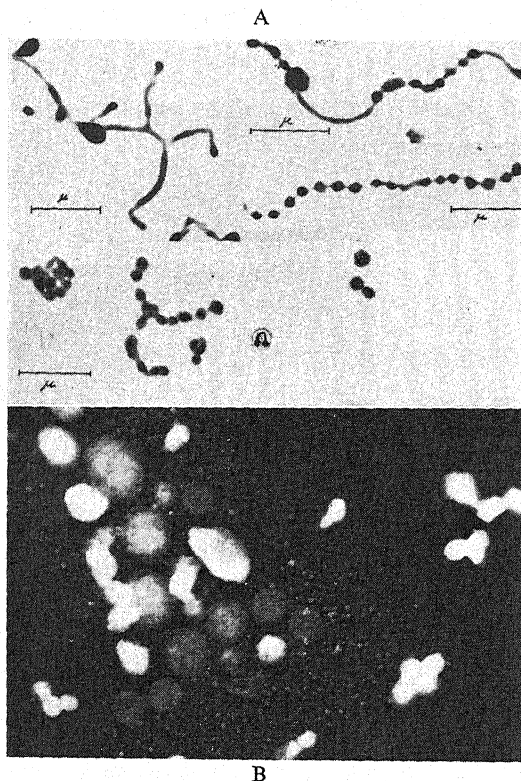


Fig. 8-1. Electronographs of pleuropneumonia organisms. *A* shows characteristic branching mycelium with terminal nodes and endomycelial condensations of protoplasm. (Photo from collection of Soc. Am. Bacteriologists, courtesy Dr. E. A. Freundt, in *Acta Path. et Micr. Scand.*, 1952, 31: 508.) *B* shows very young growth, on solid medium, of a saprophytic species (Laidlaw). These show the small filtrable elements (MRU). (Photo courtesy of Drs. E. Klineberger-Nobel and F. Cuckow, Lister Inst. Prev. Med., London.)

be derived from, and to be closely related to, certain well-recognized species of bacteria.

The term PPLO is often used (as a matter of convenience) to designate *all* of these organisms: PPO, PPLO, and L forms.

**General Properties.** The PPO, PPLO and L forms are clearly neither yeasts nor molds; neither protozoa nor algae. They are obviously neither viruses nor rickettsiae. With the possible exception of the L forms, which appear to represent stages in the developmental cycle of certain bacteria, they cannot be classified as bacteria. They possess some of the properties of all of these groups yet not all of the properties of any. Their exact position in the organic system is anomalous but seems to be near the bacteria. Because long, filamentous structures have been described in the developmental cycle of the PPO and PPLO they have often been considered as belonging to the group of mold-like bacteria, the Actinomycetales. Their classification is still under discussion. It has been suggested that they be placed in a separate order, the

Mycoplasmatales, family Mycoplasmataceae, genus *Mycoplasma*. This is probably not the end of the argument!

The distinctive characters of these three groups of organisms may be tabulated, and compared with those of some other microorganisms, as shown in Table 6.

**Cultivation.** A very favorable medium for all of these organisms is beef-heart infusion broth, pH about 8.0, containing about 25 per cent animal serum or certain lipoprotein derivatives of serum. Growth occurs best on solid media. Medium containing less than 15 per cent serum will rarely support growth of PPO or PPLO but may support bacterial L forms.

Important contributions in this field are media for PPLO of completely known composition, such as one containing *no serum*, made up of a solution of glucose, several minerals like  $\text{KH}_2\text{PO}_4$  and  $\text{FeSO}_4$ , with 15 amino acids and a vitamin.\* Another medium consists of a lipoprotein factor from serum, 9 amino acids, several vitamins, inorganic salts and several other pure substances of known composition. Agar is generally used for solidification. Tin ions appear to be requisite. Incubation is at  $37^\circ\text{C}$  for 2 to 10 days or more. The atmosphere in the plates must be saturated with moisture. PPO and PPLO are facultative with regard to oxygen. Most L forms also appear to be so.

Quantitative methods for determining rate and amount of growth are based on (a) measurements of turbidity; (b) colony diameters; and (c) enumeration of colonies.

**SELECTIVE CULTIVATION.** Most PPO and PPLO are markedly resistant to a number of substances which are completely inhibitory to all bacteria or certain large groups of them. For example, thallium acetate, sulfonamide drugs, penicillin and other antibiotics, and certain antibacterial aniline dyes are often used to suppress growth of extraneous bacteria when isolation of PPLO

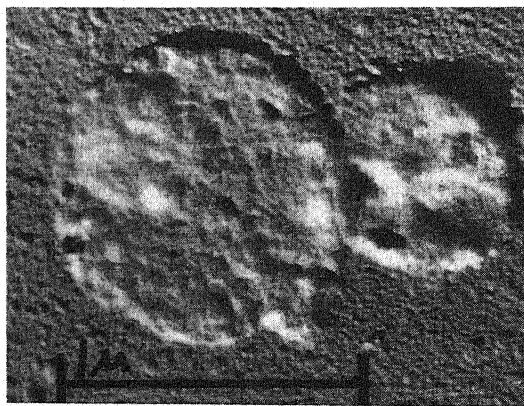


Fig. 8-2. One form of PPLO. This strain was isolated from a case of urethritis in a human being by Dr. L. Dienes. Cultivated on agar medium as described in text. ( $\times 20,000$ .) Note the relatively large, thickened granule in the larger organism. These are frequently seen. There is no evidence of a definite cell wall. (Courtesy of Drs. H. E. Morton, J. G. Lecce, J. J. Oskay and N. H. Coy, in *J. Bact.*, 1954, vol. 68.)

\* Media of completely known and reproducible composition are called *synthetic media*.

**Table 6.** *Comparison of Pleuropneumonia-like Organisms, Viruses, Rickettsiae and Bacteria.*

PROPERTY	PPO AND PPLO	L FORMS	VIRUSES		RICKETTSIAE	BACTERIA
			LARGE	SMALL		
1. Visibility*	Like large viruses	Like PPLO	Just within range of visibility	Not visible	Readily visible	Readily visible
2. Filtrability†	Have filtrable stage	Like PPLO	Pass filters with some difficulty	Readily	Not filtrable‡	Not filtrable
3. Growth on lifeless media	Growth occurs; require cholesterol and other blood derivatives	Can grow on ordinary media; prefer serum (15-20%)	No growth	No growth	No growth	Grow on simple media
4. Morphology	Very pleomorphic	Not highly pleomorphic	Not markedly pleomorphic	Not highly pleomorphic	Vary as do bacteria	Somewhat pleomorphic (Ch. 15)
5. Rigid cell wall	Absent	Absent or not clear	Not clear	None evident	Present	Present
6. Sensitivity to osmotic changes and low S.T.†	Highly sensitive	Not highly sensitive	Not clear; probably variable	Not clear; probably variable	Not clear	Not highly sensitive
7. Colonies grow into agar	Characteristically	Like PPLO	No growth	No growth	No growth	Variable; generally not highly sensitive
8. Resistance to penicillin, violet, KTe	Highly resistant	Like PPLO	Somewhat resistant to penicillin; other data limited	Resistant to antibiotics; data on others limited	Like large viruses	Only Actinomycetales
9. Relation to bacteria	None apparent; do not revert to bacteria	Are probably a developmental stage; revert to bacteria	None known	None known	None known	

\* With ordinary light microscopes.

† Through bacteria-retaining filters which pass viruses.

‡ S. T. = surface tension.

! Except *R. burnetii*, cause of Q fever.

is attempted from materials heavily contaminated with ordinary bacteria. In this respect the PPO and PPLO resemble the small viruses. Yet it has been demonstrated that they have a definite, autonomous metabolism, in this resembling the larger viruses. L forms are also highly resistant to penicillin and some other antibacterial agents but not to *all* antibiotics.

PPO and PPLO are also readily cultivable in living chick embryos, like bacteria, viruses and rickettsiae, and in media containing aqueous extracts of finely divided, whole chick embryo instead of serum. They sometimes contaminate biological materials like cultures of human tissue cells.

#### COLONIES (ALL FORMS)

Minute colonies, so small as usually to be invisible to the naked eye and visible only with a hand lens or low power of the microscope, appear along the lines of inoculation on solid media (Fig. 8-3). These true colonies must not be confused with similar-looking structures ("pseudocolonies") composed of spherocrystals and other aggregations of lifeless material which sometimes appear on the surface even of sterile serum-agar plates (Fig. 8-4). These occur as a result of various physicochemical factors acting locally, including scratching the surface of the agar with a needle. They have no relation to the inoculum. The genuine colonies are definite in outline and slightly raised and have a dark, vacuolated or granular central portion. The central density of colonies on solid medium is due to growth of the organisms below, as well as on, the surface of the agar. The central portion above the surface contains well-defined elementary bodies which may be stained in situ.

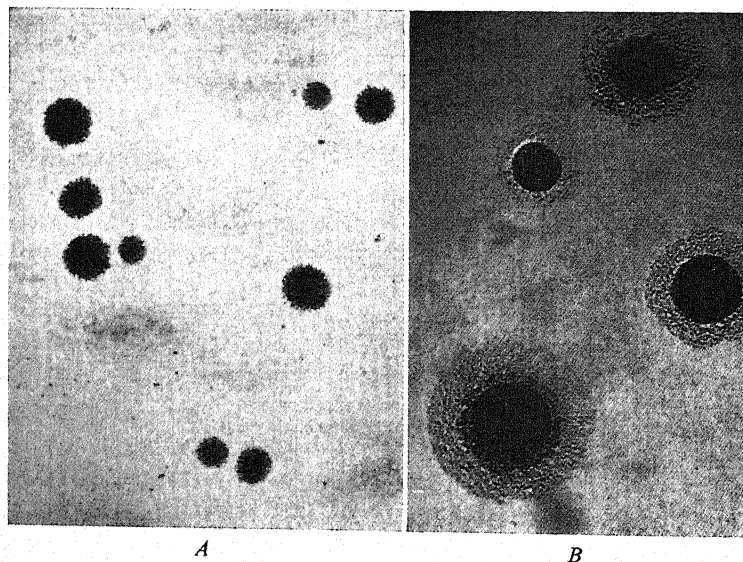


Fig. 8-3. Colonies of pleuropneumonia-like organisms (L bodies) on 20 per cent horse-serum dextrose-starch agar. (48 hours at 37° C) (× 70). Note the minute size and granular structure. *A* and *B* are from different cases of rat-bite fever, a disease caused by *Streptobacillus moniliformis*. (Brown and Nunemaker, Bulletin of the Johns Hopkins Hosp. vol 70)



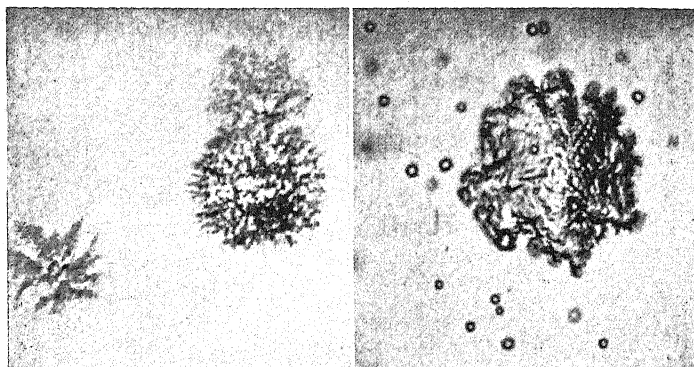


Fig. 8-4. *A*, Pseudo-colonies in 30 per cent rabbit-serum agar incubated 2 weeks at 37° C ( $\times 115$ ). *B*, similar medium incubated 6 weeks. Both media were completely sterile. (Brown, Swift and Watson, *J. Bact.*, vol. 40.)

Certain *very tiny* colony types, which appear to grow only beneath the surface, are called T-form colonies.

**PROPAGATION.** For propagation, a small agar block containing the desired colony is carefully cut out of a agar plate. It may be used to inoculate an appropriate fluid medium such as one of the synthetic media mentioned above. It is usually more satisfactory to inoculate agar on the same or another plate by sliding the piece of colony-containing agar, inverted, over the surface of the fresh medium.

**Staining and Morphology.** Like most microorganisms, the minute, colorless and transparent PPO, PPLO and L forms can be seen much more readily if they are stained with an aniline dye. A good method of staining these organisms is to cut out a block of agar on which the colonies are growing and place it upon a slide. Then invert upon it a coverslip on which is dried methylene blue-azure (deposited from alcoholic solution). The microscope is focused on the coverslip.

The elementary bodies in the central part of the colonies are seen to be minute, round or elongated forms ranging in diameter from around 0.001  $\mu$  to 7 $\mu$ . The smallest are about 1/1000 the diameter of bacteria. But this is much larger than many viruses. They may also be ring-shaped or sometimes irregular or bacillary, but always exceedingly minute.

**Life Cycle.** The fundamental units of growth in the colonies are the tiny elementary bodies. They are sometimes spoken of as *minimal reproductive units* (MRU). Electron micrography has yielded much of interest in studies of these minute organisms.

(a) **PPO AND PPLO.** In these organisms the MRU enlarge to form what are called "large bodies." Many small, new, elementary bodies develop inside. These are readily liberated by rupture of the large body since there appears to be no definite, rigid cell wall as in bacteria. The new organisms repeat the reproduction cycle. There are some variations from this simple theme but mostly they concern size and shape of the elements in the cycle. Sometimes the large bodies develop bud-like protrusions or filamentous extensions which later break up into, or liberate, small, new MRU.

(b) **L BODIES.** In bacteria with L forms, a basically similar cycle occurs.

The bacterial cell swells up and forms a "large body." Inside, there develop many L forms, much like the MRU mentioned above. They are liberated, and grow as MRU in typical, PPLO-type colonies. They may also grow directly into bacterial cells.

In developing L forms bacterial cells develop irregular protusions and undergo obscure internal developments. The cell eventually breaks apart into minute bacillary forms if the cell was a rod; spheres if it was a coccus. Some of these developmental cycles have been followed in single, individual cells by means of time-lapse, phase microphotography and appear to be fairly definite. (See Figs. 8-5 and 8-6.)

L bodies are not motile and flagella have not been seen even though the L bodies may have been derived from a motile bacterium. Internal structure of L bodies is obscure.

**Occurrence of L Bodies.** L bodies have been demonstrated in numerous species of bacteria, bacillary as well as coccal in form. They occur notably in *Proteus* species, *Bacteroides*, *Escherichia*, hemolytic streptococci, *Pasteurella*, *Neisseria*, *Streptobacillus moniliformis* (also called *Murimycetes streptobacilli-*

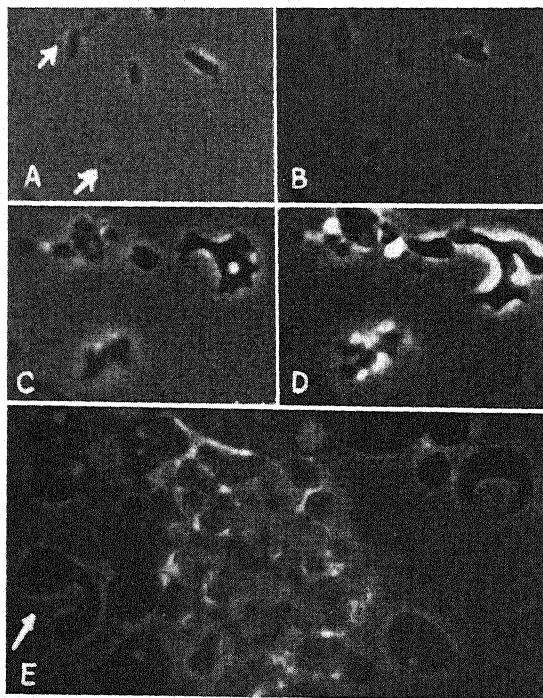


Fig. 8-5. Formation and development of L forms from a common species of bacterium (*Proteus mirabilis*). Similar to Figure 8-6 but showing the reverse process. Note especially changes in organisms marked by arrows, which produce L forms by budding. Most others swell, develop internal granules (possibly elements like MRU?) and finally develop into colonies of L forms. Growth is on agar containing 1000 units of penicillin per ml. Intervals after inoculation of agar are (Min.): A, 30; B, 165; C, 420; D, 540; E, 1775. ( $\times 1188$ .) (Courtesy of Miss Lilybett Valentin and Dr. M. A. Medill, Dept. of Botany, Univ. of Pennsylvania. Unpublished.)

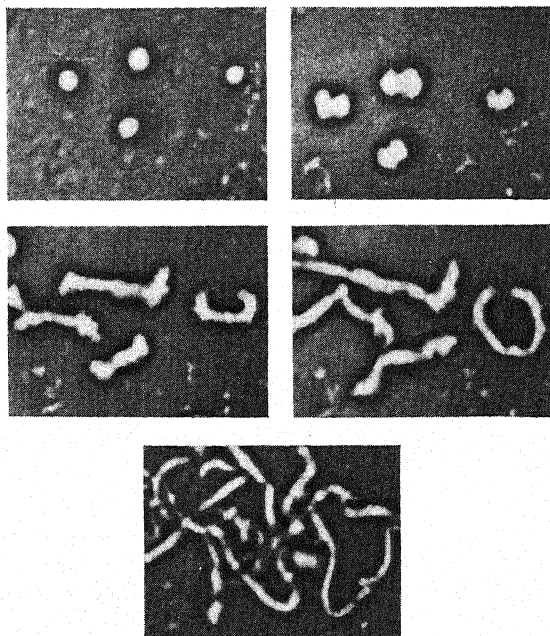


Fig. 8-6. Reversion of L forms of a common bacterium (*Proteus mirabilis*) into rod forms. The series of microphotographs of the same L forms (taken by time-lapse motion picture on Cine Kodak XX film using phase microscopy, Chapt. 9) shows successive stages in the process. Intervals after inoculation of agar are (Min.): upper left, 30; upper right, 109; lower left, 160; lower right, 186; center, 225. (About  $\times 2400$ .) (Courtesy of Drs. M. A. Medill and W. G. Hutchinson, in *J. Bact.*, 1954, vol. 68.)

*moniliformis*, *Haverhillia multiformis* and other names) and others. These are all common bacterial species, discussed farther on. In many of these bacteria the L bodies appear to develop as a response to certain unfavorable agents, especially penicillin and increased salt content of the culture medium. They grow on the same media and under the same cultural conditions as the parent bacteria, and are antigenically (chemically) similar to the parent bacteria.

**Relations of PPO, PPLO and L Forms.** Because of these curious similarities it seems permissible to regard PPO, PPLO and L forms as closely related. L forms may provisionally be regarded as stages in the developmental cycle of recognized species of bacteria. PPO and PPLO may be thought of as representing similar developmental cycles in which, due possibly to a higher degree of parasitic evolution, the clearly bacterial stage has disappeared or become rudimentary.

**Streptobacillus Moniliformis.** One of the most interesting of the L organisms is generally referred to as "L<sub>1</sub>," following the nomenclature of Klieneberger who first described it in 1935. It is present in nearly all cultures of the very pleomorphic, filamentous bacterium usually called *Streptobacillus moniliformis*.

*S. moniliformis* and the L<sub>1</sub> organisms grow well under the same conditions as other L forms. *S. moniliformis* may be obtained from pus of the ears of rats where it frequently causes otitis media, and from the blood of human

beings suffering from rat-bite fever, one type of which it also causes. It grows on meat-infusion media, especially with blood or serum added. The colonies, superficially examined, are much like those of ordinary bacteria. Smears show bacillus-like rods and also filaments in which branching is common. Swellings, globules and granulations of the cells are a prominent feature as the colonies age.

After several days of incubation of the colonies of *S. moniliformis*, dense areas appear under them. By washing away the earlier growth of *S. moniliformis* with broth, the denser portions are found to remain adherent to the agar. These are the growths of the L<sub>1</sub> elements. The agar containing these elements may be cut out, macerated in broth and used to inoculate new agar or broth, as described earlier in this chapter. On agar media the organisms retain their L<sub>1</sub> form; in broth the filamentous growth of *S. moniliformis* reappears.

The elementary granules can pass bacteria-retaining filters, and *S. moniliformis* colonies will develop from such filtrates.

#### REFERENCES

- Abrams, R. Y.: A method for the cultivation of L forms in liquid media. *J. Bact.*, 1955, 70:251.
- Allbritten, F. F., Sheely, R. F., and Jeffers, W. A.: *Haverhillia multiformis* septicemia. *J.A.M.A.*, 1940, 114:2360.
- Carter, G. R.: Pleuropneumonia-like organisms isolated from bronchopneumonia of cattle. *Science*, 1954, 120:113.
- Cuckow, F. W., and Klieneberger-Nobel, E.: Further studies of organisms of the pleuropneumonia group by electron microscopy. *J. Gen. Micro.*, 1955, 13:149.
- Dienes, L.: Isolation of L type cultures from *Bacteroides* with the aid of penicillin and their reversion into the usual bacilli. *J. Bact.*, 1948, 56:445.
- Dienes, L., and Sharp, J. T.: The role of high electrolyte concentration in the production and growth of L forms of bacteria. *J. Bact.*, 1956, 71:208.
- Dienes, L., and Weinberger, H. J.: The L forms of bacteria. *Bact. Rev.*, 1951, 15:245.
- Edward, D. G., and Freundt, E. A.: The classification and nomenclature of organisms of the pleuropneumonia group. *J. Gen. Micro.*, 1956, 14:197.
- Heilman, F. R.: A study of *Asterococcus muris* (*Streptobacillus moniliformis*). I and II. *J. Infect. Dis.*, 1941, 69:32 and 45.
- Klieneberger-Nobel, E.: Filtrable forms of bacteria. *Bact. Rev.*, 1951, 15:77.
- Medill, M. A., and Hutchinson, W. G.: The reversion of the L form of *Proteus mirabilis* into the rod form. *J. Bact.*, 1954, 68:89.
- Medill, M. A., and O'Kane, D. J.: A synthetic medium for the L type colonies of *Proteus*. *J. Bact.*, 1954, 68:530.
- Morton, H. E., Lecce, J. G., Oskay, J. J., and Coy, N. H.: Electron microscope studies of pleuropneumonia-like organisms isolated from man and chickens. *J. Bact.*, 1954, 68:697.
- Morton, H. E., Smith, P. F., and Keller, R.: Prevalence of pleuropneumonia-like organisms and the evaluation of media and methods for their isolation from clinical material. *Am. J. Pub. Health*, 1952, 42:913.
- Robinson, L. B., Wichelhausen, R. H., and Roizman, B.: Contamination of human cell cultures by pleuropneumonia-like organisms. *Science*, 1956, 124:1147.
- Sabin, A. B.: The filtrable microorganisms of the pleuropneumonia group. *Bact. Rev.*, 1941, 5:1,331.
- Shepard, M. C.: T-form colonies of pleuropneumonia-like organisms. *J. Bact.*, 1956, 71:362.
- Smith, P. F.: Amino acid metabolism by pleuropneumonia-like organisms. *J. Bact.*, 1955, 70:552.
- Smith, P. F.: Synthetic media for pleuropneumonia-like organisms. *Proc. Soc. Exp. Biol. & Med.*, 1955, 88:628.

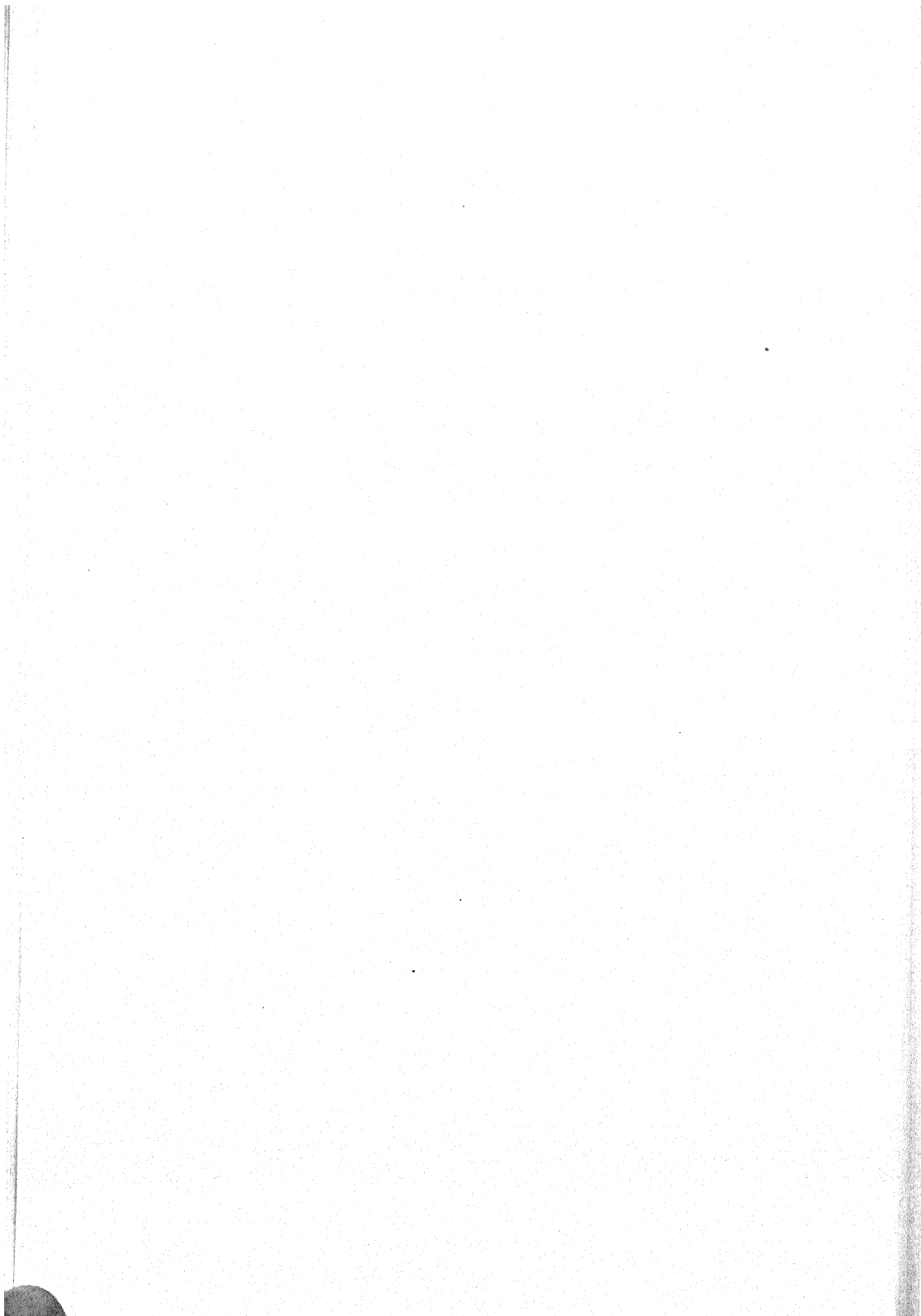
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- Smith, P. F.: Quantitative measurement of the growth of pleuropneumonia-like organisms. *Appl. Micro.*, 1956, 4:254.
- Smith, W. E., Mudd, S., and Hillier, J.: L-type variation and bacterial reproduction by large bodies as seen in electron micrographic studies of *Bacteroides funduliformis*. *J. Bact.*, 1948, 56:603.
- Idem: Electron micrograph studies of two strains of pleuropneumonia-like (L) organisms of human derivation. *Ibid.*, p. 589.
- Stempen, H.: Demonstration of a cell wall in the large bodies of *Proteus vulgaris*. *J. Bact.*, 1955, 70:177.
- Turner, A. W.: Study of morphology and life cycles of the organism of pleuropneumonia contagiosa bovis. *J. Path. and Bact.*, 1935, 41:25.
- Warren, J.: Observations on some biological characteristics of organisms of the pleuropneumonia group. *J. Bact.*, 1942, 43:211.
- Wittler, R. G., Cary, S. G., and Lindberg, R. B.: Reversion of a pleuropneumonia-like organism to a *Corynebacterium* during tissue culture passage. *J. Gen. Micro.*, 1956, 14:763.
- Yamamoto, R., Adler, H. E., and Cordy, D. R.: The propagation of a virulent goat pleuropneumonia-like organism in the chick embryo. *J. Bact.*, 1955, 69:472.

## SECTION 2

# *Methods and Phenomena of Microbiology*

SECTION 1 of this book gave a synoptic view of microorganisms in general. The student learned about the size, structure, activities, and particularly the major properties which distinguish microorganisms of each of eight great groups from one another. In this section more details are given. The student acquires a working knowledge of microbiology. He learns about microscopic methods, more of the structural peculiarities, how one handles microorganisms in the laboratory, how they grow, their biochemical characters, physiology, etc. Bacteria are used as the basis for most of this discussion because they have been very thoroughly studied and because they are easily manipulated and controlled in the laboratory. What is true of bacteria is, in general, true of most other living cells, with modifications necessitated by environments of various kinds. Where contrasts occur as, for example, between bacteria and algae or protozoa or viruses, these are pointed out. All of this information is of basic importance to the student of microbiology, regardless of his anticipated field of specialization. The student thoroughly familiar with the contents of sections 1 and 2 will have little trouble with the remainder of the book and should find the subject of growing interest. The more proficient we become in any game or intellectual activity, the more pleasurable we find it; even chess, bridge, or ice hockey!



## Optical Methods in Microbiology

### LIGHT MICROSCOPY

FOR MAKING "enlarged" images, simple lenses like "magnifiers" or "reading glasses" have been in use for centuries, especially in ancient China. These are forms of *simple* microscopes. Such simple magnifiers had definite limitations due to their crude construction. About 1590 a Dutch spectacle maker, Zacharias Janssen, used a second lens to magnify the image produced by a primary lens. This is the basic principle of the compound microscope used by every microbiologist today. Galileo invented an improved compound microscope in 1610. In attempts to see still more, better microscopes have been built, until today we possess complicated, compound instruments which give magnifications up to 3000 diameters; ten times that of the ancient, simple instruments. (Fig. 9-1.)

### THE COMPOUND MICROSCOPE

**General Description.** A complete description of the optics of compound microscopes will be found in any good, modern, college textbook of physics. As shown in Figure 9-2, light is reflected upward by the adjustable mirror at the foot of the instrument. The light is *refracted* in the condenser, just above, so as to bring the rays to a central point with as little diversion from the vertical (and consequent loss) as possible at the plane of the object. The condenser is equipped with an iris diaphragm which is used to eliminate excessive light and reduce the aperture of the condenser for various lenses. Usually the light rays emerge from the top side of the condenser as a cone of light with the apex downward. The rays which are not too divergent pass through the object. If they do not diverge at too wide an angle they enter the first and most "powerful" lens of the microscope: the objective. The wider the scope (or numerical aperture or N.A.) of this lens (or the wider the angle it can survey) the more of the divergent rays (those at the periphery of the cone of rays) it can bring in.\*

\* A good camera lens, for example, is a wide-angle lens, and brings in much light (it is "fast"). Such lenses have N.A. of around 2.5 and a relatively short focal length:  $1\frac{1}{2}$  to 2 or 3 inches. An oil-immersion objective lens has a focal length of only a few millimeters and a N.A. of around 0.9. It is a very "short-sighted" lens with limited N.A. and works only a millimeter or two from the object in order to get as many of the divergent rays from the object within its narrow scope as possible.



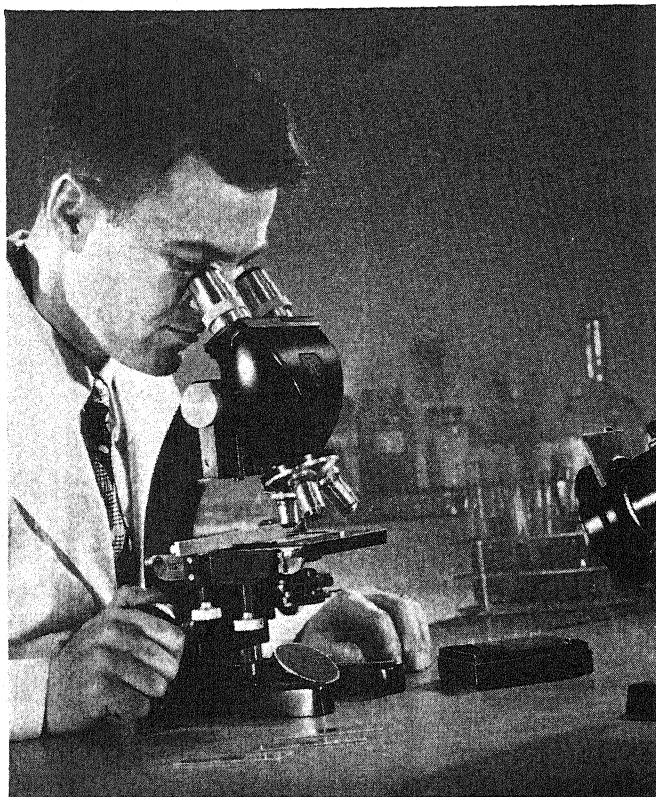


Fig. 9-1. Method of using a high-power microscope. This is a binocular instrument (B and L TBV-8) but, except for two eyepieces, is constructed fundamentally like a monocular instrument. Note the natural position of the head, lack of facial strain, position of the hand on the coarse adjustment and the location of the slide under the low-power lens. (Courtesy Bausch and Lomb Optical Co., Rochester, New York.)

**Oil Immersion.** As the rays of light emerge from the upper surface of the condenser, some (especially peripheral rays) are refracted beyond the scope of the objective and lost. Others are reflected away from the underside of the glass slide on which the object is mounted, and lost. Others are refracted from its upper surface. Others are lost by refraction and reflection in the object, and at the surface of the objective lens. A considerable part of these various losses and distortion of the light rays can be prevented by eliminating the optical effect of most of these surfaces. This is done by placing a clear, colorless fluid (immersion oil), having the same refractive index as glass, between condenser and slide and between slide and objective lens; hence the use of oil immersion lenses as objectives for high-power microscopy. Immersion oil, in effect, can increase the N.A. of a lens because it brings in more light rays.

Several lenses, close above the objective, are used to correct difficulties inherent in such objectives, namely, spherical and chromatic aberration (distortions of image due to lens curvature, etc.).

**Real and Virtual Image.** The *real image* produced by the objective is brought

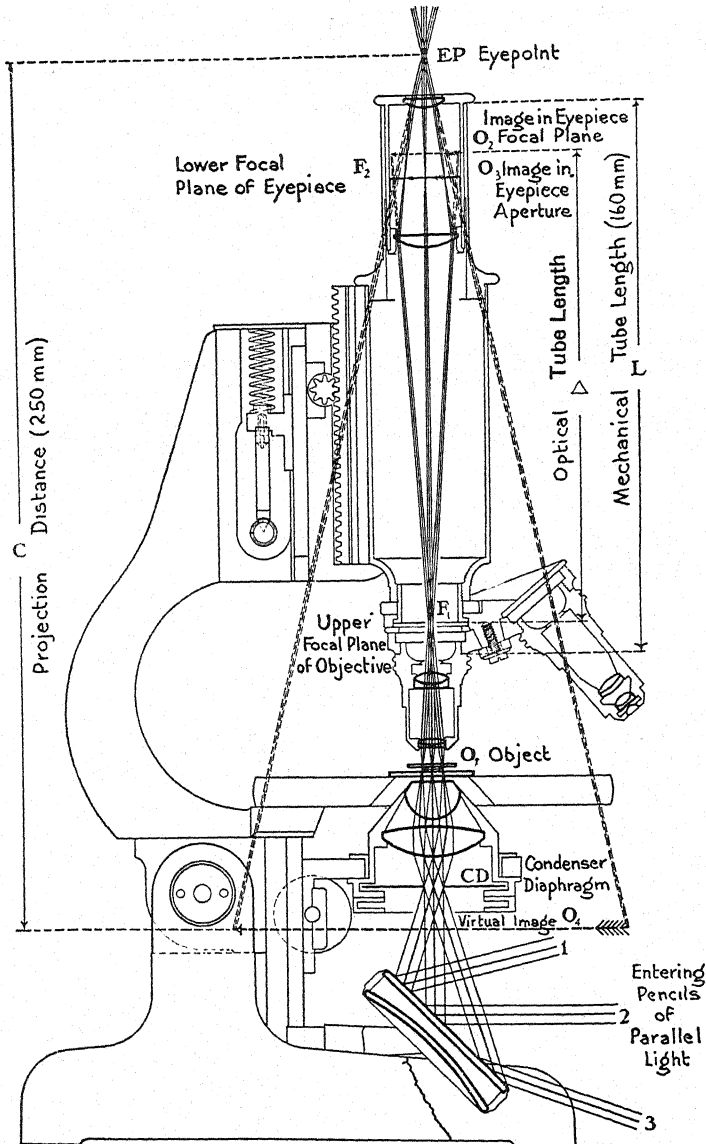


Fig. 9-2. Chart showing path of light through laboratory microscope. (Courtesy Bausch & Lomb Optical Co., Rochester, N. Y.)

The entering pencils of light (1, 2, 3) are reflected upward by the mirror through the diaphragm (CD) opening. In passing through the condenser lenses they are focussed on the object (O<sub>1</sub>) on a glass slide. Passing through the object they are refracted by the lenses in the objective. They are brought to a focus at, and then diverge from, the upper focal plane of the objective (F<sub>1</sub>). Refracted by the lower lens of the eyepiece they form a series of real and virtual images (O<sub>3</sub>, O<sub>2</sub>) in the eyepiece focal plane (F<sub>2</sub>) and eyepiece aperture. These are magnified by the upper lens of the eyepiece and focussed at the eyepoint (EP). The object on the glass slide is then seen by the eye as an enlarged, virtual image (O<sub>4</sub>) which seems to be at a level a little below the condenser.

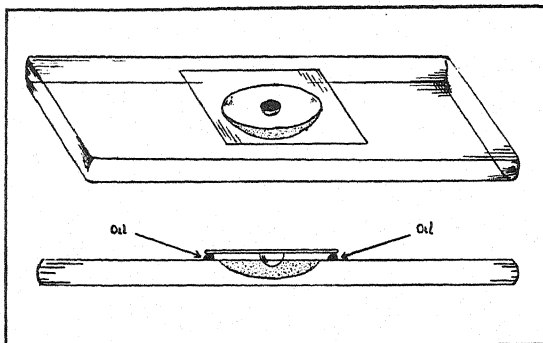


Fig. 9-3. Hanging drop preparation. The size of the oil droplets is exaggerated.

to a focus *within* the eyepiece. This is a system of 2 or 3 lenses near the top of the microscope. This real image is magnified by the topmost lens (ocular) and appears as a greatly enlarged *virtual image*, seeming to be projected to a position just above the reflecting mirror.

**Resolving Power.** The real measure of a microscope is its *resolving power*. This is based on the fact that the sensory endings (receptors) of the optic nerve in the retina of the eye have dimensions of several  $\mu$ . If light rays from *two* tiny objects, very close together (say  $0.2 \mu$ ) strike *one* of these retinal receptors, the image conveyed by the optic nerve will be that of only *one* object. The two objects blur together. The unaided eye does not perceive that the objects are separate; i.e., it does not *resolve* them.

By magnification, resolution is increased but, as we shall see later, there are definite limitations to resolution by ordinary microscopes (around  $\times 1200$ ) due to the nature of visible light rays. An oil immersion objective lens magnifying the object about 90 to 100 diameters is generally used. The ocular or eyepiece should magnify the real image, formed by the objective, 10 to 12.5 diameters. The total magnification of between 900 and 1125 diameters thus obtained is most useful, higher magnifications tending to give "fuzzy" outlines due in part to poor resolution.

**"Hanging Drop" Preparations.** Bacteria in a natural, living state are best viewed when suspended in a clear fluid of some sort; usually water, saline solution or broth. The cells are transparent, colorless and refractile and so tiny that they are often difficult to find and even to identify as bacteria in the drop of fluid. This is especially true of the spherical types (cocci).

To observe bacteria in this state it is necessary to put a loopful\* of the fluid in which they are suspended in the center of a thin cover slip. On each of the four corners place a tiny droplet of mineral oil. Hold a "hollow-ground" slide, depression down, over the drop and bring the two into contact. Invert the slide quickly so that the drop cannot run off to one side. A *tiny* additional drop of clear mineral oil may now be run under the edges of the cover slip at each corner if needed. This spreads under the cover slip and prevents drying of the drop (Fig. 9-3).

\* By a "loop" is meant the space included by a tiny ring or loop made at the end of a thin wire. The wire is fixed into some sort of handle so that it may be sterilized in a flame. Loops are usually about 2 mm. in diameter and a loopful is a small drop.

In the hanging drop we may see the size, shape and arrangement of bacteria and their motion if they are motile. Sometimes bright, refractile granules and spores may be seen within the living cells. Due to the fact that bacterial cells contain no chlorophyll and are colorless and transparent it is extremely difficult to study them since this lack of color prevents details from showing clearly. Further, when suspended in fluid, they move about, either because of their own motility, currents in the fluid, or brownian movement. The observation of bacteria in hanging drop preparations, therefore, yields limited though valuable information.

**Smears for Staining.** To overcome this difficulty, Koch's device of immobilizing bacterial cells and coloring them with aniline dyes is generally used. In order to accomplish this, the material containing the bacteria (soil, urine, pus, milk, feces, saliva) is diluted with water, saline solution or broth. A small drop of the suspension is spread evenly in a thin film, with a loop, over an area about 1 cm. in diameter. This film is commonly called a "smear."

**Nature of Dyes.** Most of the dyes used in microbiology may be thought of as salts of two kinds: (1) *acidic*; those in which the color-bearing ion (called the *chromophore*) is the anion, e.g., sodium<sup>+</sup> eosinate<sup>-</sup> (the dye, eosin); (2) *basic*; those in which the chromophore is the cation, e.g., methylene blue<sup>+</sup> chloride<sup>-</sup>. The first type of dyes is acidic in the sense that, as an acid, the chromophore combines with a base (NaOH) to form the dye salt; the second is basic because the chromophore acts like a base, combining with an acid (HCl) to form the dye salt.

In general, acidic dyes combine more strongly with cytoplasmic (basic) elements of cells; the basic dyes with nucleic (acidic) elements of cells.

Some dyes do not depend on forming salts or chemical combinations with the stained material. They merely coat the surface by the process of adsorption, or they may merely dissolve or precipitate in the surrounding substrate. Probably both physical and chemical interactions occur during staining and probably different processes are involved in staining different species. None is *fully* explained or understood.

### STAINING BACTERIA

**Simple Stains.** For general bacteriological work basic stains are most used, because bacteria react toward stains as though composed principally of nucleic acid, which takes basic dyes most readily. Methylene blue, safranin, crystal violet, basic fuchsin, thionin, etc., are very widely used. All belong to the group of aniline (coal tar) dyes. Any simple aniline dye solution may be applied by flooding the smear with it. Löffler's methylene-blue solution\* is very widely used and reveals many details of form and structure. Other solutions and colors are also available for special purposes. The dye is allowed to remain in contact with the smear for about one minute and is

\* Löffler's stain (Soc. Amer. Bact. "Manual of Methods").

#### Solution A

Methylene blue.....	0.3 gm
Ethyl alcohol (95 per cent).....	30.0 ml

#### Solution B

KOH (0.01 per cent).....	100.0 ml
Mix solutions A and B. The mixture keeps well.	

then washed off with a gentle stream of cool water. The slide is then blotted (*not rubbed*) between two pieces of filter or blotting paper and when dry is ready for examination with the oil immersion lens as previously described.

Now, a simple stain such as Löffler's is of great value for many purposes. But another staining procedure, devised by the Danish scholar Gram, is more valuable because it enables us to differentiate between kinds of bacteria which may be of different species yet of the same general form and size. It is therefore called a *differential stain*.

✓ **Gram's Stain.\*** To the smear which, for purposes of discussion, we shall assume to contain a variety of bacteria (e.g., saliva), crystal violet solution† is applied for thirty seconds. This is gently rinsed off and an iodine solution‡ is applied for thirty seconds. This, in turn, is rinsed off. Ninety-five per cent ethyl alcohol is applied and renewed until all but the thickest parts of the smear have ceased to give off dye. (This usually takes from twenty seconds to one minute.)

The *differential* feature of the method is now apparent. Examination with the microscope will reveal the fact that, as Gram found, while many bacteria retain the violet-iodine combination, others will have yielded it largely to the alcohol and are almost as nearly invisible as before. Those species of bacteria which *retain* the stain are called *gram-positive*. Those which *yield* it to the alcohol are called *gram-negative*.

But the staining process is not yet complete. There is still the important final step of applying the *counterstain*§—a dye of some contrasting color, usually eosin (red), safranin (red), brilliant green or Bismarck brown. Any one of these dyes colors the gram-negative species and they become as visible as the gram-positive ones, but are readily differentiated by their color.

Thus, by applying Gram's stain, which takes but five or six minutes, we can learn a great deal about any bacterium. We make visible not only the form and size and certain other structural details, but we can also at once assign the organisms which are present in the material being examined, to one of two great artificial groups of bacteria: the gram-negative or gram-positive. As will be seen later, this is very helpful in identification procedures.

\* Gram's stain (Hucker's modification, Soc. Am. Bact. "Manual of Methods").

†

#### Solution A

Crystal violet (85 per cent dye content).....	2.0 gm
Ethyl alcohol (95 per cent).....	20.0 ml

#### Solution B

Ammonium oxalate.....	0.8 gm
Distilled water.....	80.0 ml

Dilute solution A about 1 in 5 with distilled water and mix with an equal volume of solution B.

‡ **Lugol's iodine**

Iodine.....	1 gm
Potassium iodide.....	2 to 5 gm
Distilled water.....	300 ml

Allow to stand 24 hours for the iodine to dissolve. It may be necessary to add a few more crystals of potassium iodide.

§ **Counterstain:**

Safranin (2.5 per cent solution in 95 per cent alcohol).....	25 ml
Distilled water.....	75 ml

It should be noted, however, that some organisms are "borderline" cases in respect to Gram's stain, sometimes being positive, other times negative; and sometimes both positive and negative cells are seen in the same culture. As a rule, repeated tests will reveal the true nature of the bacterium. Often slight variations in cultural conditions or staining technique will affect the result. For example, many bacteria are not definitely gram-positive unless cultivated in the presence of at least 5 per cent blood or serum. The acidity or alkalinity (pH) of the fluid in which the bacteria are suspended will also markedly affect their reaction to the Gram stain. It has been suggested that bacterial species can be arranged in a gradient based on their reaction to the Gram stain. In this gradient the strongly and constantly gram-positive species take up and hold measurably more crystal violet than the permanently and definitely gram-negative species. Gram-variable species hold variable, intermediate amounts.

**MECHANISM OF THE GRAM STAIN.** If we could learn just *why* some organisms are gram-positive and others gram-negative, we could perhaps clarify questions surrounding the nature and distribution of the nucleus of bacteria, the alterations in Gram staining properties of an organism under different conditions of growth, the structure of the cell wall or cell membrane, and vital questions on bacterial physiology. The actual mechanism, though investigated for years, is still not clear. The property of gram-positiveness appears to reside in the cell wall. In most gram-positive bacteria the cell wall appears to have some special affinity for the crystal-violet-iodine complex. The cell walls of gram-negative bacteria do not have this affinity. In general, it seems that the crystal violet and iodine penetrate both gram-positive and gram-negative cells. In most gram-positive cells the dye and iodine form a compound in the cell wall which is relatively insoluble in the decolorizing agent (usually alcohol) and which passes out from the cell wall with difficulty. Removal of various components of the cell wall (magnesium ribonucleate, lipids, carbohydrates, etc.) by various agents (ribonuclease, hot water, ether, etc.) make the cell gram-negative. That the cell wall plays a decisive role in the Gram stain is shown by the fact that mechanical disruption of gram-positive cells makes the entire cell gram-negative. No part of the disrupted cell retains gram-positiveness.

**CORRELATION OF GRAM REACTION WITH OTHER PROPERTIES.** Whatever the explanation of the Gram reaction it is important to note that there are certain characteristic differences between most gram-positive and gram-negative bacteria. Several of these are shown in Table 7. There are exceptions to some of these generalizations but it is evident that the property of gram-positiveness is related to very fundamental physiological properties. The reasons are not entirely clear.

**Ziehl-Neelsen Stain.** Another differential stain is that of Ziehl-Neelsen. It is used especially for staining tuberculosis bacilli (*Mycobacterium tuberculosis*) and related organisms (genus *Mycobacterium*) having the chemical composition peculiar to these bacilli, i.e., an abundance of a particular waxy material in the cell. Such organisms are gram-positive but this method does not give as useful information about them as the Ziehl-Neelsen or "acid-fast" stain.

In using this stain a smear of the material to be examined is made as usual,

**Table 7.** *Some Differences Between Gram-positive and Gram-negative Bacteria.*

PROPERTY	GRAM-POSITIVE	GRAM-NEGATIVE
Susceptibility to sulfonamide drugs and penicillin	Marked	Much less
Inhibition by basic dyes like crystal violet	Marked	Much less
Susceptibility to low surface tension	Marked	Much less
Susceptibility to anionic detergents	Marked	Much less
Digestion by trypsin or pepsin (dead cells)	Marked	Much less
Dissolved by 1% NaOH	Marked	Not resistant
Ratio of RNA to DNA in the cell (approx.)	8:1	Almost equal

dried, and fixed by heat. The smear is then flooded with a special solution of carbol fuchsin and heated to 90.0° C, over a steam bath for four minutes. This softens the wax and the dye supposedly penetrates. After washing off the excess dye, the smear is treated for five minutes with cold 95 per cent alcohol containing 5 to 10 per cent hydrochloric acid. Presumably, the wax retains the red dye in spite of the acid-alcohol, which removes the color from everything else. Organisms retaining the red stain are said to be *acid-fast*. If, now, methylene blue or brilliant green be applied as a counterstain, the acid-fast bacilli stand out as bright red objects in a blue or green field. The Ziehl-Neelsen stain\* is a *differential* stain because it differentiates acid-fast organisms from nonacid-fast ones (Fig. 9-4).

**MECHANISM OF THE ACID-FAST STAIN.** Acid-fastness, like gram-positiveness, disappears with physical disruption of the cell. It has been suggested that acid-fastness is a matter of relative solubilities. For example, the red dye, fuchsin, is more soluble in phenol than in water or acid-alcohol. Phenol, in turn, is more soluble in lipids or waxes, such as are present in tubercle bacilli, than in water. In the acid-fast staining procedure, the phenol, with red fuchsin in it, leaves the water-alcohol of the carbol-fuchsin dye mixture and enters the cell lipids in which it is more soluble. Here it remains because it is here more soluble than in the decolorizing agent (acid-alcohol) and because the intact cell membrane prevents lipids from leaving the cell to dissolve in the de-

\* Ziehl-Neelsen acid-fast stain (carbol fuchsin)

Solution A

Basic fuchsin.....	0.3 gm
Ethyl alcohol (95 per cent).....	10.0 ml

Solution B

Phenol (melted crystals).....	5.0 ml
Distilled water.....	95.0 ml

Mix solutions A and B. The mixture keeps well. A counterstain of Löffler's methylene blue is generally used, although some workers use brilliant green or saturated aqueous solution of picric acid for better contrast.

colorizer. If the cell membrane is broken, the lipids leave the cell and the acid-fast property disappears.

**Negative Staining.** As generally used, this is not really a method of staining bacteria, but of staining the background a solid black, usually with nigrosin.\* This dye fails to penetrate the bacteria at all, and leaves them unstained to appear as light areas in the darkened field (Fig. 9-5). Only the outlines of the organism are made apparent by this method, and consequently it has a limited application, being used mainly for such forms as cannot be stained by any of the ordinary methods, especially spirochetes.

A source of error in this method is shrinkage and distortion of bacterial cells during the drying process. This often makes them look larger than they are.

In addition to the various stains described above, there are others designed to bring out special details such as spores, capsules, flagella and so on. Description of each of these methods will, however, be reserved to the discussion

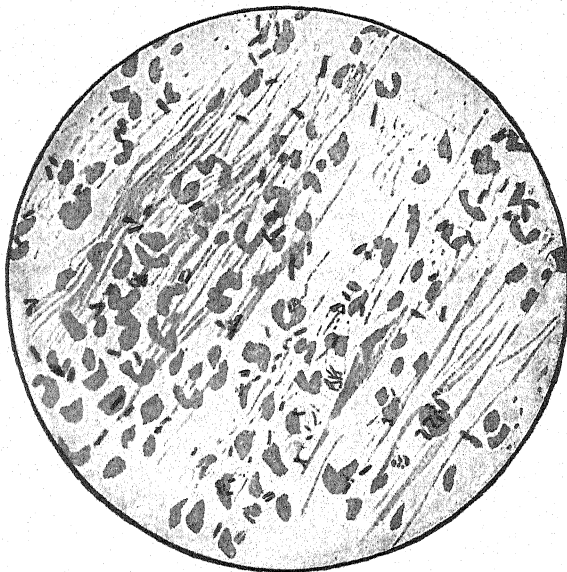


Fig. 9-4. Tubercle bacilli in sputum. Ziehl-Neelsen ( $\times 900$ ). The bacilli have been stained red. The mucus and pus cells in the sputum are stained blue for contrast. Note the granular appearance of some of the bacilli and their arrangement in clusters. The many bacilli pictured here are in a very tiny fraction of a droplet. (Cornet and Meyer.)

\* **Dorner's method for negative staining**

(Grease-free slides must be used)

Nigrosin.....	10 gm
Water.....	100 ml

Boil 30 minutes and add 0.5 ml formalin. Filter through paper and store in 2 ml amounts in sterile tubes. Place a loopful of the suspension to be examined on a slide. Immediately add an equal amount of the nigrosin solution, mix, and spread out in a thin film. (Thick films will crack and peel.)

Dry in air. Do not wet the slide. Do not heat it!



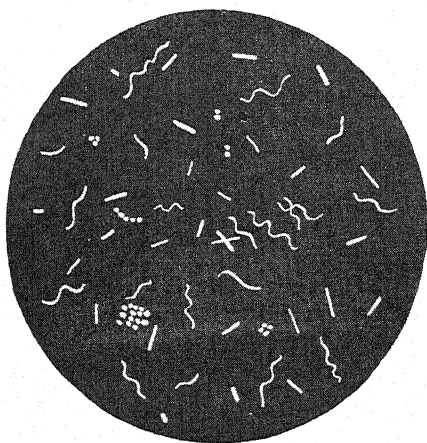


Fig. 9-5. Appearance of various bacteria as seen, when prepared by negative staining method ( $\times 900$ ).

of the structural features of the bacteria to which it applies and to which we shall give some attention in a later chapter.

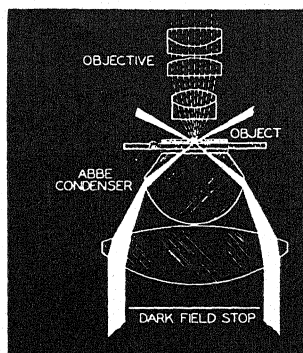
**Darkfield Method.** A method analogous in principle to the “negative staining” procedure is that of darkfield illumination. The ordinary compound microscope may easily be equipped for this work by substituting a darkfield condenser (purchasable from microscope manufacturers) in place of the Abbé condenser.

In a darkfield condenser an opaque stop is arranged to *prevent* the entrance of any *direct* rays of light from the mirror *straight upward* into the tube of the microscope. All *peripheral* rays are, however, reflected *obliquely* to the *center* of the upper surface of the microscope slide, emerging from the upper surface of the slide at such an angle that they do not enter the objective lens unless some object be present to *reflect* them *upward* (Fig. 9-6). The empty field, therefore, appears dark. When a fluid containing any particles such as dust, bacteria or spirochetes is placed on the slide at the focal point of these oblique rays, each particle becomes visible as a brightly illuminated speck due to the light reflected upward from its surface into the barrel of the microscope (Fig. 9-7). The remainder of the field appears dark, hence the term *darkfield*. Unlike the process of negative staining, the darkfield shows not only outward form but motility, since the organisms, covered with a cover slip, are examined in a moist, *living* state. As outward form and motility are the chief means by which certain important microorganisms, especially spirochetes, are distinguished, the method is very valuable in types of work where such species are involved; for example, diagnosis of syphilis.

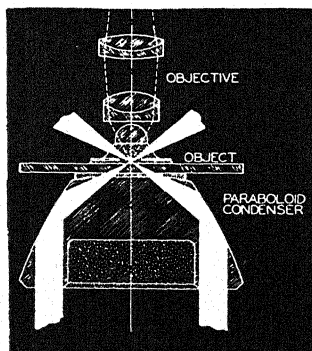
### ELECTRON MICROSCOPY

After the discovery of tobacco mosaic virus by Iwanowski in 1892, it became apparent that there are creatures so small as to make bacteria appear enormous by comparison. These tiny creatures cannot be seen even with the most highly developed lens system depending on *visible* light.

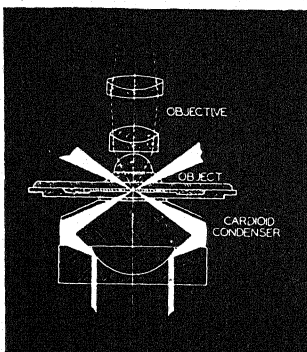
The difficulty lay not with the lensmakers but in the fact that the wave length of visible light is around 6000 Å (Å = angstrom unit = 0.000,000,1 mm.). Particles with a diameter of less than one half of this wave length, or



Abbé Condenser with Darkfield Stop



Paraboloid Condenser



Cardioid Condenser

Fig. 9-6. Various forms of condensers for oblique illumination of the darkfield. In the upper picture some object on the slide is reflecting light up through the objective lens. Note that only peripheral light rays pass the condenser. (Bausch and Lomb Optical Co.)

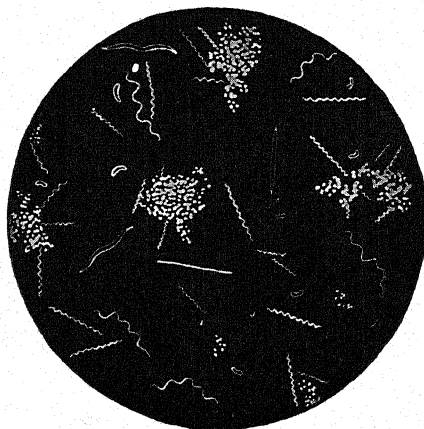


Fig. 9-7. Darkfield preparation ( $\times 900$ ). This picture shows a group of spirochetes and other bacteria from a lung abscess. Note that the thicker bacteria are seen only as luminous outlines. This is because they are visible only by means of light reflected from their outer surfaces.

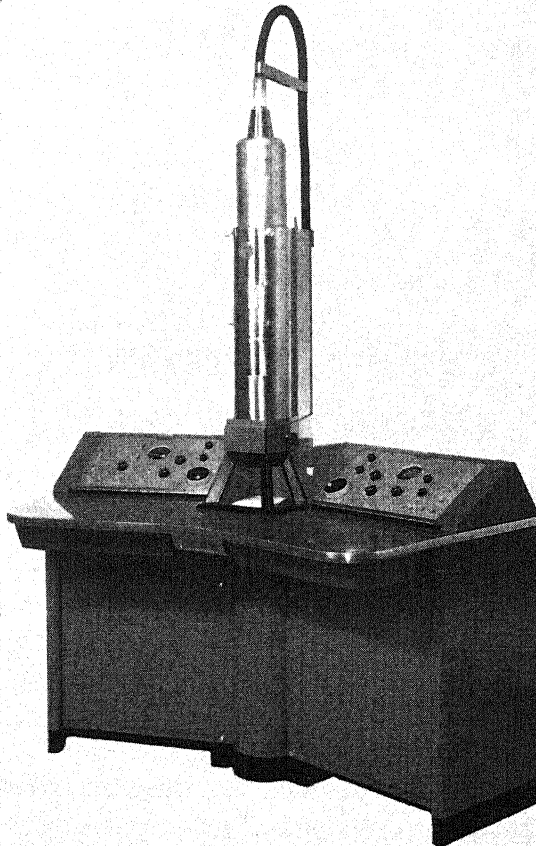


Fig. 9-8. This electron microscope, developed by the Radio Corporation of America, permits viewing of particles smaller than one 10-millionth of an inch in any diameter. It provides magnification 50 per cent greater than any heretofore possible. This magnification is so enormous that a human hair would take on the dimensions of the Lincoln Tunnel. (Courtesy of Radio Corporation of America, Camden, N. J.)

lines separated by a distance less than about  $2000 \text{ \AA}$ , cannot be "resolved," that is, they are not perceptible as separate particles because light waves around  $6000 \text{ \AA}$  length entirely "skip over" the individual particles, just as a man might not be aware of pebbles in his path because of the length of his stride.

While it has long been known that electromagnetic radiations such as x-rays have very short wave lengths, the preparation of glass lenses or reflectors for electron beams is impossible because glass is opaque to electrons. However, it was observed that electrons are deflected from their line of propagation by magnetic fields and, second, it was found that electrons moving with the speed imparted by 60 KV have a wave length of only about  $0.05 \text{ \AA}$ , or  $1/100,000$  that of visible light. The latter discovery made plain the possible usefulness of electronic waves in microscopy, while from the former it was clear that circular magnetic fields could be used for "refracting" electron

beams, much as a lens refracts light rays, forming electron images in the same manner that visible light images are formed by lenses.

From these two basic discoveries the modern electron microscope (Fig. 9-8) has evolved. The units in this instrument are nominally analogous with units in an ordinary compound microscope but deal with electron beams rather than light rays. The diagram and legend in Figure 9-9 show the general

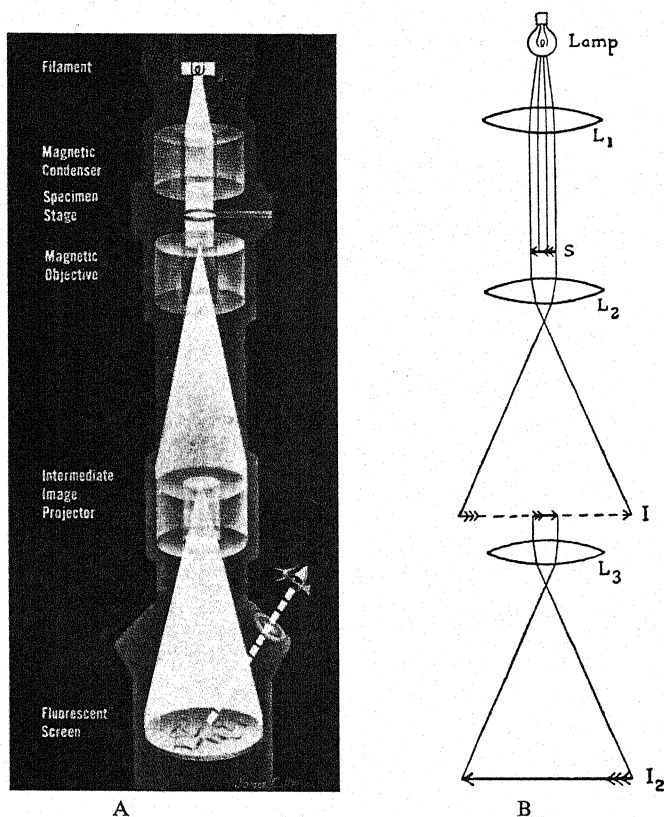


Fig. 9-9. Diagrammatic outline of the path of electron beams in the RCA electron microscope (A) as compared with the path of light rays in an ordinary light microscope (B). The similarity of the two is clearly evident. In A a beam of electrons traveling from the filament at high velocity correspond to the light rays from the lamp in B. The electron rays (A) or light rays (B) are focussed on the specimen (S in B) by condensing systems. In A this is a circular electromagnetic condenser; in B it is the lens system  $L_1$ . After passing through the specimen the magnetic objective lens coil (corresponding to the objective lens system,  $L_2$ , of the light microscope) forms a first image (corresponding to I in B), enlarged about 100 times. The magnetic intermediate image projector (corresponding to the ocular lens system,  $L_3$ , of the light microscope) then magnifies this image about 250 times (corresponding to the virtual image ( $I_2$  in B), producing an overall magnification of 25,000. The final enlarged electronic image can be viewed directly by causing it to strike a fluorescent screen which makes it visible (corresponding to the eye of the observer using a light microscope), or it can be made to record the image on a photographic plate for permanent record and for enlargement up to 100,000 or more. (A, courtesy of the Radio Corporation of America; B, courtesy of the Society of American Bacteriologists collection, print 31.)

arrangement of the focusing systems and other parts of an electron microscope compared with those of a light microscope. The electron source is a tungsten filament at 30–100 KV potential. The electrons enter the magnetic condenser (analogous to an ordinary microscope condenser) and are converged on the specimen. After passing through the specimen, the magnetic objective-lens coil focuses them into a first (real) image, enlarged about 100 times. The magnetic intermediate-image projector then magnifies the real image about 250 times, making an over-all magnification of 25,000. The final, enlarged image can be viewed directly by causing it to strike a fluorescent screen which makes it visible. The image can also be thrown upon a photographic plate for permanent record. Portions of the photographs may be enlarged 4 times without undue loss of detail, thus giving a picture 100,000 times as large as the object. This degree of magnification is almost inconceivably great. A vague notion of it may be gained by imagining the page on which this is printed to be enlarged to the same degree. It would become a slab of spongy material as thick as a 2-story building is high, over 8 miles wide and about  $13\frac{1}{2}$  miles long. A human hair magnified to this degree would take on dimensions approximating those of the Holland Tunnel. A good comparison of electron microscopy with ordinary microscopy is seen in Figure 9–10.

Several details of the operation of the instrument are of interest. Because the motion of electrons is impeded by air, the interior of the microscope must be maintained at a vacuum by means of suitable pumps. This necessitates air locks for the insertion and removal of objects and photographic plates. The operator can look into the main tube, by means of portholes, and see the images on the fluorescent screens, manipulate the object, make suitable adjustments of field strength of the focusing magnets, alignment, etc. The object is mounted on an extremely thin (0.000001 cm. thick) cellulose film, since glass slides would be opaque to electrons.

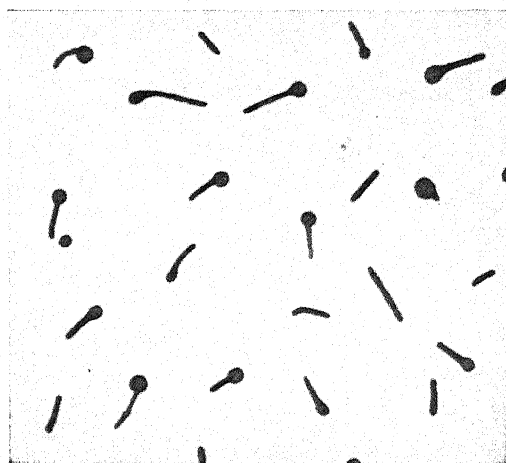
Under conditions of electron microscopy living organisms cannot survive and physiological processes in live cells cannot be studied by this means at present.

A word of caution may be spoken in closing our discussion. The organisms to be viewed in an electron microscope must be dried and this produces shrinkage and distortion. In addition, many important biological structures may contain little or no matter which obstructs the electrons and so cast no shadows and remain invisible when electrons are passed through them. In order to visualize detail, it is necessary to produce contrasts in the object. This may be done in one way by selective staining (as phosphotungstic acid). Then some parts of the object are made more “opaque” to the electrons than others.

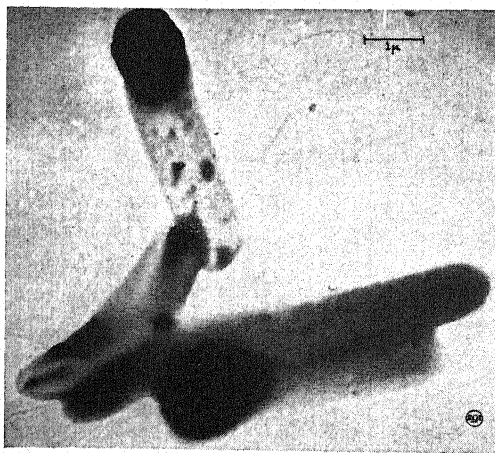
Artifacts having the most deceptive appearances, and very suggestive of living things, are found in lifeless materials (see Figure 9–11). The objects seen in photographs of electronic images are merely shadows, much like shadows on an x-ray plate, and depend for visibility only on degree of opacity, to electrons, of parts of the object. They may represent actual structures, or mere thickenings of the object, or artifacts. In any case, our interpretations of them should be extremely guarded until much more is known of electronic microscopy and its pitfalls.

**Shadowing Technique.** One of the most valuable techniques for the microbiologist is that called electron shadow-micrography. This is best described

by the inventors as "depositing an exceedingly thin (ca.  $7\text{ m}\mu$ ) layer of metal (chromium or gold) obliquely (in vacuo) on the preparation . . . High points on the surface intercept more than an average number of the condensing atoms (of metal) and at the same time shield areas immediately behind them. These shielded areas, devoid of deposited metal, appear as 'shadows' of the elevated regions responsible for them. Just as an observer . . . in a plane sees many details of a landscape at sunrise, or sunset, which would be invisible to him at noon, so metal shadow-casting brings out much detail that is not to be seen in an unshadowed (or in a vertically coated) preparation." The method applied to viruses is wonderfully revealing (Fig. 9-12). Many other interesting



A



B

Fig. 9-10. Two pictures of the lockjaw organism (*Clostridium tetani*) as seen magnified  $\times 2000$  by ordinary microscope (A) and magnified  $\times 14,000$  by electron microscopy in B. The terminal spores are seen as large dark bodies. (A, Ford, Textbook of Bacteriology; B, S. Mudd and T. F. Anderson, J.A.M.A., 1944.)

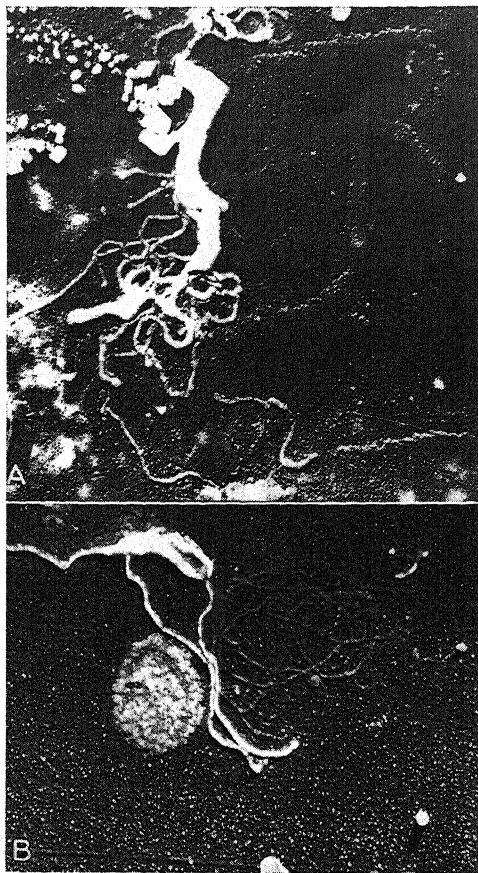


Fig. 9-11. *A*, unwashed preparation from sterile tissue fluid. Filaments do not arise from a cell-like image here, but from a long, thick grouping of closely-packed, adjoining NaCl crystals. Near the side of the picture some of the crystals simulate virus-like particles ( $\times 14,000$ ). *B*, isolated filaments and fibers from sterile tissue fluid. They have the same appearance as those observed during disintegration of erythrocytes. Many small particles as well as a large one resembling elementary bodies of some viruses are seen in the background ( $\times 14,000$ ). (Photo courtesy of Dr. J. H. L. Watson, Edsel B. Ford Institute for Medical Research, Detroit, Mich., 1950, vol. 60.)

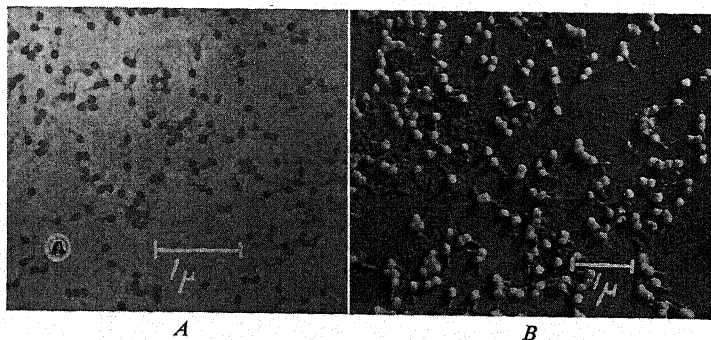


Fig. 9-12. Electronographs of a virus (bacteriophage). *A*, without metal shadowing; *B*, with metal shadowing. (Courtesy of Dr. D. Gordon Sharp, Duke University.)

"shadowed" pictures have been made, showing, for example, the details of structure and size of poliomyelitis virus, tobacco mosaic virus and several species of bacteria.

### FLUORESCENCE MICROSCOPY

By fluorescence is meant the property of reflecting rays having a wave length different from that of the incident rays. Thus, substances having a certain color by ordinary light appear of a totally different color by ultraviolet light. Objects invisible when "illuminated" only by ultraviolet light may become brilliantly luminous if painted with a fluorescent substance such as quinine sulfate (fluoresces violet in ultraviolet light) or the dye auramine (fluoresces yellow in ultraviolet rays). By staining bacteria with a fluorescent dye and observing them in a field illuminated only with ultraviolet light, they can be made visible as luminous objects readily seen in a black field and differentiated from nonfluorescent objects.

One adaptation has been in the study of tuberculosis. The fluorescent dye, auramine, has a strong affinity for wax-like substances in tubercle bacilli. When the bacilli are stained with auramine, and examined in the dark by ultraviolet light, they fluoresce with a luminous yellow light.

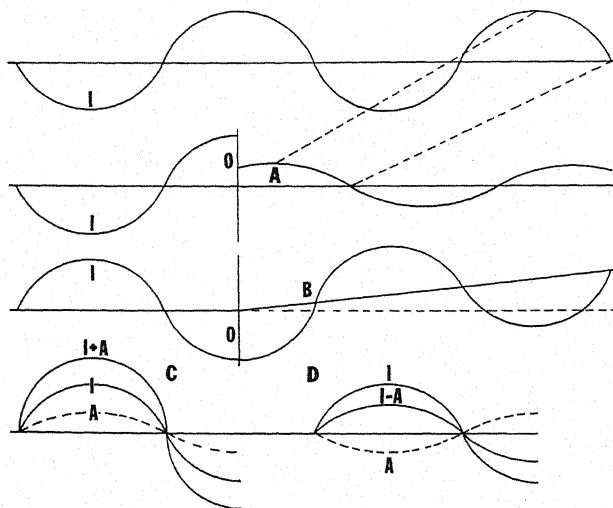


Fig. 9-13. Relation of incident light waves (*I*), transmitted waves (*A*) and diffracted waves (*B*) in phase microscopy. Retardation in phase (broken lines) and dimming (reduction of amplitude) of *A* after passing through the object (*O*) are shown in comparison with *I*. These differences create contrast between object and surround. However, these differences are not readily perceptible to the unaided eye. The effects of adjusting the difference in phase between *A* and *I* by means of the phase-altering plate in the microscope are seen in *C* as additive (resonance) (bright contrast), and in *D* as subtractive (interference) (dark contrast). This optical manipulation of phase differences produces contrast between object and surround which is readily perceptible to the eye when the various waves are brought to a focus as an image. *B* shows waves not passing *through* the object but diffracted in passing near it. For purposes of this discussion *B* and *I* are considered as one because diffraction (*B*) plays a small role in phase contrast as compared with change of phase (*A*).



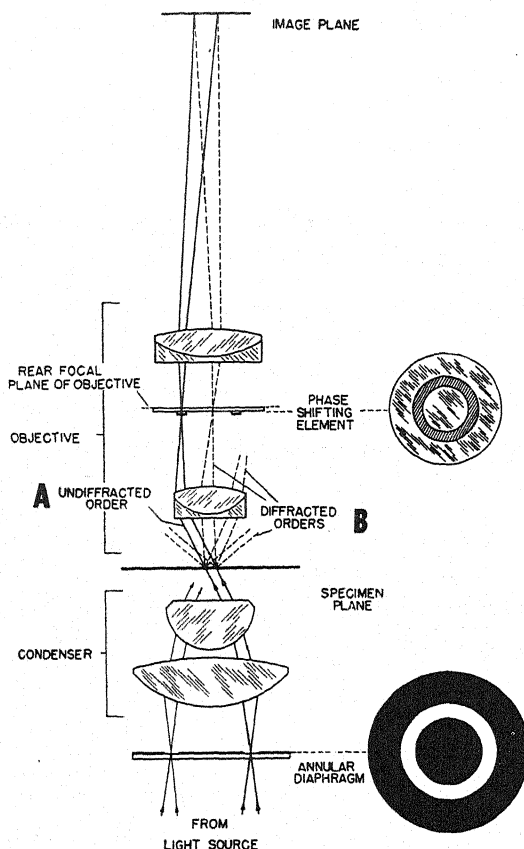


Fig. 9-14. Image formation by phase contrast. An *annular aperture* in the diaphragm, placed in the focal plane of the substage condenser, controls the illumination on the object. The aperture is imaged by the condenser and objective at the *rear focal plane* of the objective. In this plane the *phase-shifting disk* (diffraction or phase plate), is placed.

With the particular plate shown, light waves *A* (solid lines) are *transmitted* through the object and pass through the phase-altering ring on the phase plate. At this point they acquire a one-quarter-wave-length advance over light waves *B* (broken lines) which do not pass through the object but are partly *diffracted* around it. Waves *B* do not pass through the wave-altering ring on the phase plate. The resultant *interference* or *resonance* effects of the two portions of light form the final image. Altered *phase* relations in the illuminating rays, induced by otherwise invisible elements in the specimen, are translated into brightness differences (*Contrast*) by the phase-altering plate; hence phase contrast. (Courtesy of Dr. J. R. Benford, Bausch and Lomb Scientific Bureau.)

### PHASE MICROSCOPY

If one examines the smaller microorganisms, such as bacteria, in their living state suspended in a hanging drop of fluid, not only is it difficult to see the organisms but it is next to impossible to discern clearly any of the internal structures. This is due to the fact that there is almost no difference in refractive index between protoplasmic structures and their surrounding fluids. The situation is almost like trying to see a tiny fragment of ice in a bowl of water. However, in biological materials, the slight differences that *do* exist can, by

means of special optical devices, be greatly enhanced so that readily perceptible contrast (phase contrast) is produced between these objects and their surroundings. This may be described briefly as follows.

A special, ring-shaped diaphragm is used in place of the usual iris diaphragm, and a light-wave-altering filter is placed back of the objective lens. The light waves passing *through* the object are dimmer than, and *out of phase* with, incident waves (I), and diffracted waves (B) (Fig. 9-13). The difference in phase and intensity between transmitted wave and non-transmitted waves are intensified by the filter in the objective lens and are seen by the eye in distinct outlines of light and dark phase contrast. The relations of the phases and amplitude of two light waves are shown in Figure 9-13. The optical arrangements are shown in Figure 9-14.

#### REFERENCES

- Bartholomew, J. W., and Mittwer, T.: The Gram stain. *Bact. Rev.*, 1952, 16:1.
- Benford, J. R.: The Theory of the Microscope. Bausch & Lomb Optical Co., Rochester, N. Y., 1952.
- Bennett, A. H., Osterberg, H., et al.: Phase Microscopy. John Wiley and Sons, New York, 1951.
- Conn, H. J.: The History of Staining. Biotech Publications, Geneva, N. Y., 1933.
- Conn, H. J.: Biological Stains. 6th ed. Geneva, N. Y., 1953.
- Conn, H. J., Bartholomew, J. W., and Jennison, M. W.: Staining Methods. Manual of Methods, Pure Culture Study of Bacteria, 11th ed. Leaflet IV. Biotech Publications, Geneva, N. Y., 1954.
- Cosslet, V. E.: Practical Electron Microscopy. Academic Press, New York, 1951.
- Fischer, R. B.: Applied Electron Microscopy. Ind. Univ. Press, Bloomington, Ind., 1953.
- Hall, C. E.: Introduction to Electron Microscopy. McGraw-Hill Book Co., New York, 1953.
- Lamanna, C., and Mallette, M. F.: Basic Bacteriology. Williams & Wilkins Co., Baltimore, Md., 1953.
- Libenson, L., and McIlroy, A. P.: On the mechanism of the Gram stain. *J. Inf. Dis.*, 1955, 95:22.
- Newton, B. A.: The properties and mode of action of the polymyxins. *Bact. Rev.*, 1956, 20:14.
- Oster, G., and Pollister, A. W.: Physical Techniques in Biological Research: I. Optical Techniques. Academic Press, Inc., New York, 1955.
- Reynolds, F. W., and Hesbacher, E. U.: Darkfield microscopy. *J. Ven. Dis. Inform.*, 1950, 31:17.
- Richards, O. W.: Phase microscopy 1954-1956. *Science*, 1956, 124:810.
- Richards, O. W., and Miller, D. K.: An efficient method for the identification of *M. tuberculosis* with a simple fluorescence microscope. *Am. J. Clin. Path.*, 1941, 11:1.
- Richards, T.: The use of the microscope; I, II, III. *Laboratory Practice*, London, 1955, 4:488; 1956, 5:56.
- Various Authors: Manual of Methods for Pure Culture Study of Bacteria. Biotech Publications, Geneva, N. Y.
- Vickers, A. E. J.: Modern Methods of Microscopy. Butterworth, London, 1956.
- Werkman, C. H., and Wilson, P. W.: Bacterial Physiology. Academic Press, New York, 1951.
- Zernike, F.: How I discovered phase contrast. *Science*, 1955, 121:345.

## Morphology and Structure of Bacteria

### MORPHOLOGICAL TYPES OF BACTERIA

AS SOON as microscopes and microscopy were sufficiently well developed to permit detailed studies of microorganisms, it was found that three main forms of bacteria may be differentiated: the spherical, the cylindrical or rod-like, and the spiral or helicoidal (Fig. 1-4).

**Spherical bacteria** are called cocci (singular = *coccus*; derived from a Greek word for berry). Cocci are classified in several groups on the basis of the manner in which they cling together after fission. First, there are *diplococci*, which remain in pairs. Obviously these divide in one plane. Then there are the *streptococci* which, like the diplococci, divide in one plane but which cling together in chains, looking much like strings of beads. Other cocci divide irregularly in three planes, and cling together in masses shaped like bunches of grapes. These are called *micrococci*. Still others divide regularly in three planes at right angles to each other, forming cubical groups. These are named *sarcinae* (Latin for packet). Some cocci divide at right angles but in only one plane. They form groups of four, called tetrads. Since they were first described by Gaffky, they are called *Gaffky*.

The spherical form of cocci is often distorted by various influences, so that oval, elliptical, conical and other modifications are frequently seen.

**Cylindrical or rod-like bacteria** are called bacilli (singular = *bacillus*; a Latin word meaning "little stick" or rod). Bacilli are straight, cigarette-shaped or sausage-shaped organisms. The cell wall appears to be a fairly rigid tube or inelastic sac since growth occurs predominantly lengthwise and variations in size affect mainly the long axis. Some are curved. Sometimes bacilli are so short that the length is no greater than their diameter. These are often referred to as *cocco-bacilli*. It is difficult to distinguish them from cocci. However, in *pure* cultures this is not such a great problem, since a few of the cells are usually long enough to reveal the bacillary nature of the culture.

One other form, which may be thought of as derived from the cylindrical, is the *fusiform* bacillus, shaped like a long, thin cigar pointed at both ends.

**Helicoidal or spiral bacteria** are shaped like a coiled wire spring, ranging from a portion of a coil to 15 or more complete helices. Two main groups may be recognized: the *flexible* spiral bacteria (classified as the order Spirochaetales and commonly called spirochetes), and the *rigid* spiral bacteria

(classified in the family Spirillaceae of the order Eubacteriales). A subgroup of the Spirillaceae comprises species which are so short as to consist of only part of a spiral turn; in fact they are shaped much like a slightly twisted comma. These are grouped in the genus *Vibrio* of which *Vibrio comma* (cause of Asiatic cholera) is an important member.

The relations of the morphological types are shown in the following outline:

1. Coccus forms (spherical or derived from spheres)
  - Diplococcus*\* (pairs)
  - Streptococcus* (chains)
  - Micrococcus* (irregular clusters)
  - Gaffkya* (tetrads)
  - Sarcina* (cubical packets)
2. *Bacillus* or rod forms (straight, rod-like or cylindrical).
  - (Fusiform bacillus)
3. Helicoidal forms
  - Spirochetes (flexible; several complete spiral turns).
  - Spirillum* (rigid; 1 or more spiral turns)
  - Vibrio* (rigid; less than 1 spiral turn)

As bacteria grow they are subject to many influences, such as surface tension, osmotic pressure, nutrition and rate of growth, which affect their size and shape, much as various factors affect the size and shape of potatoes or peppers. Age also plays its role.

Referring to the straight, rod-shaped, cylindrical forms, it should be pointed out that the term "bacillus" was originally employed for all. However, when endospores† were discovered by Cohn in 1875 in the *strictly aerobic* organism now known as *Bacillus subtilis*, he differentiated sporulating aerobic organisms from other cylindrical bacteria and adopted the term *Bacillus* as a genus name. For this reason, the term *Bacillus*, capitalized and printed in italics, is now employed only for aerobic, rod-shaped bacteria which produce endospores. In general, the term "bacillus" and "bacterium" are often used interchangeably for all rod-shaped cylindrical forms. The term "bacterium" is also used in a general sense to mean any organism belonging to the class Schizomycetes.

#### SIZE OF BACTERIA

The drawings and reports by Leeuwenhoek (Fig. 2-4) gave men an idea of the size and form of certain common types of these organisms.

Bacteria are inconceivably small. Their minute size may be emphasized by various comparisons. For example, it is estimated that a cubic inch would hold nine trillion (9,000,000,000,000) cells of a medium sized bacillus (*Salmonella typhi*). It is common practice to magnify bacteria 900 to 1000 diameters with microscopes. They then look no bigger than a period on this page, or an exclamation point (!). A man magnified to the same degree would be over a mile high and 500 yards wide.

Specifically, the size of bacteria ranges around a diameter of about  $1.0\mu$

\* Terms in italics are titles of genera named for the morphology of the organisms.

† Endospores are oval, refractile, thick-walled, protoplasmic bodies which develop inside certain species of bacterial cells. Only one is formed in each cell. It represents a dormant stage which, like arthrospores of molds, is resistant to drying, heat, disinfectants, etc. Spores will be discussed more fully later.

and a length, in the cylindrical forms, around  $8\mu$ . Lengths of 200 to  $500\mu$  are not unknown, however, in certain species. Diameters usually do not exceed about  $2\mu$ .

**Size and Fission.** The size of the individual bacterial cell bears an important relationship to the nutrition of the cell and possibly to cell fission. This is because the volume of the cell increases many times more rapidly than the surface area. The volume of a cylindrical cell increases as the square of its radius ( $\pi r^2 \times \text{alt.}$ ); the volume of a spherical cell increases as the cube of its diameter ( $\text{diam.}^3 \times 0.5236$ ). Yet foods for the inner parts of either cell must pass through the cell surface since there is no mouth. As the spherical cell grows there arrives a critical point in the ratio of volume (which must be fed) to surface (which supplies food) when the favorable initial relationship between surface and content must be renewed. This is accomplished by fission, which increases the ratio of surface to volume and results in the formation of two small daughter cells. These grow and divide and so the process continues. When fission (as sometimes occurs) proceeds much more rapidly than cell growth, then the size of an individual cell diminishes as the numbers increase.

The amount of foodstuffs which can diffuse in, and the amounts of waste products which can diffuse out, through the cell coverings, have definite limitations determined by the nature of the cell wall, cell membrane, etc.

Whether wastes, nutritive requirements, reproductive cycle or other factors initiate the stimulus for cell fission is not known. Possibly all of these factors play a combined role.

The evolution of cylindrical cells, straight or spiral, with diameters more or less fixed by radially rigid (or at least unexpandable) cell walls may be regarded as a step toward efficiency and more complex organisms. In a bacterial or mold cell or filament the axial center of the protoplast\* is never more than a micron or so from the source of food supply and the means of waste disposal. The length and branching can extend (with or without division into cells) indefinitely with no loss to the protoplast.

## BACTERIAL STRUCTURE

We may consider five anatomical divisions, proceeding from exterior inward: (1) flagella, (2) capsule and/or slime layer, (3) cell wall, (4) protoplast, with (a) protoplast membrane (equivalent to cytoplasmic membrane); (b) cytoplasm (including various cytoplasmic inclusions, granules, etc.); (c) nuclear structures (see Fig. 10-12).

**1. Flagella.** Flagella are long, very fine threads of complex protein attached to the cell in various locations, the number and position being characteristic, according to the species (see Figs. 10-1 and 2). The point of origin of flagella is difficult to determine. Many electronographs indicate a cytoplasmic origin of flagella. It probably differs in different species.

**FLAGELLA AND MOTION.** *Motion of flagella* appears to be associated with a tiny granule at their point of insertion into the cell. This is seen clearly in electronographs. Some workers regard flagella merely as twirls of gummy, capsular polysaccharide which swirl and exhibit wavy movements passively as the bacteria move by some other mechanism, possibly twisting on their

\* All of the material within the cell wall.

long axis. This view is not widely held. The mechanism by which flagella are activated is not clear.

Flagella propel the bacteria by spiral waves along the length of the flagella. Not all bacteria possess flagella. Most non-flagellate species are non-motile. The power to move by means of flagella, like the power of spore formation, appears to have evolved mainly with elongation of form. Very few (if any) spherical bacteria have flagella or form spores.

In addition to the swimming motion produced by flagella there are some non-flagellate species which move slowly by creeping, as do some algae. Some of the spirochetes appear to move without flagella but in others flagella appear to have been demonstrated.

**BACTERIAL MOTILITY.** Whatever their mechanism of motility, motile bacteria can move only in fluids. They cannot leave a fluid surface. They may pass through the air, but not of their own volition. It is only as passengers or riders on particles of dust in the air, or on objects like pencils, eating utensils, books, etc., or in droplets such as those given off by a person when sneezing or coughing, or in streams of water, airplanes, ships or trains that they can travel great distances.

Their speed is sometimes very great when measured by their size. Some of them cover a distance equal to scores of times their length in a second. An automobile would have to speed at a hundred miles an hour to accomplish the same thing. Some bacteria move very slowly and sedately, sometimes almost imperceptibly. It has been estimated that the energy required to move a bacterium through an aqueous medium is of the order of 56 electron volts per second; not great horsepower!

**DEMONSTRATION OF MOTILITY.** Motility is easily visible by direct observa-

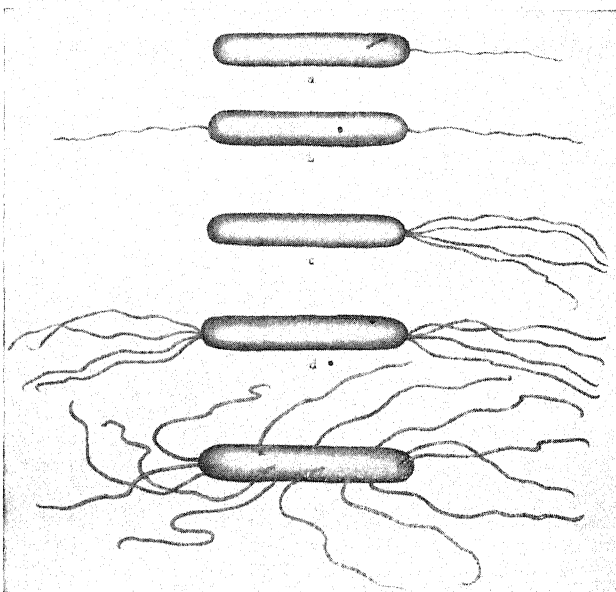


Fig. 10-1. Types of flagellation: *a*, monotrichate; *b*, amphitrichate; *c* and *d*, lophotrichate; *e*, peritrichate.

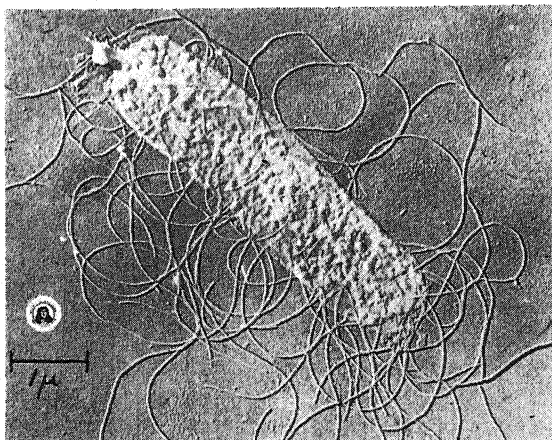


Fig. 10-2. Electronograph of a common bacterium (*Proteus vulgaris*) showing peritrichous flagellation. Note the fineness of the flagella, their apparent insertion through the cell wall of the bacterium and the apparent origin of several flagella in a small granule, suggestive of a blepharoplast. Note also the diffuse, granular character of the cell contents. (From the collection of the Society of American Bacteriologists, courtesy of Drs. A. L. Houwink and C. F. Robinow and Miss W. van Iterson.)

tion of motile bacteria in a droplet (hanging drop) of the fluid in which they are living. In any culture of bacteria, especially old cultures, motile cells may be difficult to find among thousands of dead or senescent cells. Young cultures should always be used. In cultures acidified by fermentation, bacteria lose their motility. Strict anaerobes lose motility on contact with air.

It is necessary to distinguish carefully between true motility and brownian movement. Truly motile bacteria progress definitely and continuously in a given direction. Brownian movement is a purposeless oscillation within a very limited area. It is due to molecular forces entirely external to the bacteria. Unless the bacteria are fairly active, brownian movement is sometimes rather difficult to distinguish from true motility.

**DEMONSTRATION OF FLAGELLA.** Bacterial flagella easily become detached and are so fine as to be visible only when stained, or delineated with the electron microscope (Fig. 10-2).

A useful means of staining flagella is that recommended by Leifson.\* It must be pointed out that students will ordinarily not find it easy to stain flagella on the first try. The factors involved are not all understood. However,

**\* Leifson's method for staining flagella**

Stain: Solution:	I. NaCl .....	1.5% in distilled H <sub>2</sub> O
	II. Tannic acid .....	3% in distilled H <sub>2</sub> O
	III. Pararosaniline acetate.....	0.9 gm
	Pararosaniline hydrochloride....	0.3 gm
	(or 1.2 gm certified basic fuchsin)	
	Ethyl alcohol .....	100 ml

Mix I, II and III in equal proportions and hold in tightly stoppered bottle at about 4° C.

On smear prepared as noted above quickly place 1 ml of stain. Leave at room temperature for 7-15 minutes. (Try several smears; various intervals). Rinse generously but gently with tap water—do not pour off stain first. Counterstain with diluted methylene blue will often improve the results.

a little perseverance is usually rewarded with success. Essentially the method consists of precipitating tannic acid on the flagella. The tannic acid acts as a mordant; i.e., an agent which causes the stain to stick to the stained object.

**FORMS OF FLAGELLA.** There may be several forms of flagella: coiled, curly, normal, or wavy. Several forms may occur on one cell, and even on one flagellum. The type of curvature may be partly due to distortion during drying and processing incident to staining, and to other extraneous factors. However, the wave lengths and amplitudes of these coils (or curves or waves) appear to be fairly constant and to bear some significant relationship to each other and possibly to species. For example, the wave length of a curly flagellum may be one half the wave length of a wavy or normal flagellum on the same cell. This may be distinctive of the species (see Fig. 10-3).

**2. Capsule (Slime Layer, or Sheath).** Outside of the cell wall proper there appears to be, on most bacteria, a layer of slimy, viscous or gummy substance varying in thickness and consistency according to species, growth conditions, phase of variation, or genetic characters which will be discussed later on. The *chemical composition* of the capsule varies from species to species (or variety) but appears to be a remarkably constant and distinctive characteristic of that species or variety, governed by genetic mechanisms. The electron microscope has aided greatly in the study of the slime layer or capsule, especially in those species in which it is a very thin sheath. Some organisms on which a capsule is not discernible may nevertheless possess the same chemical substance, in a very thin layer on the cell surface, as determined by immunological (serum) tests. It appears to be closely linked with the cell and may be inapparent merely because it is not formed in great quantity. These surface complexes

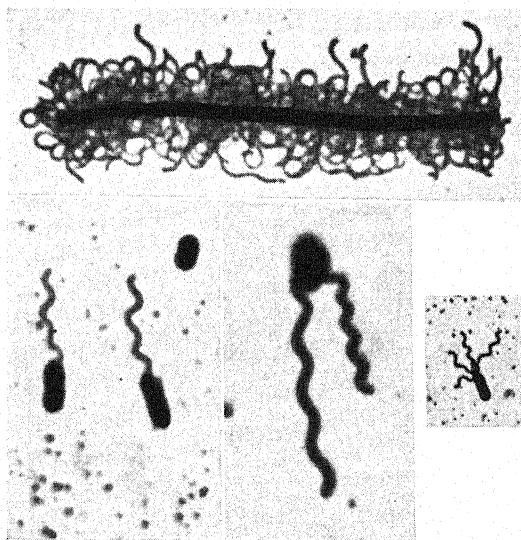


Fig. 10-3. Bacteria with flagella stained with Leifson's flagella stain. Various arrangements of flagella are seen. Note that in the bacillus with two flagella the wavelength in one flagellum is twice that in the other. Knowledge of the wavelengths of flagella is assuming importance as a means of diagnostic identification. Magnification about  $\times 3000$ . (Courtesy of Dr. Einar Leifson, Stritch School of Medicine, Loyola University, Chicago, Ill.)



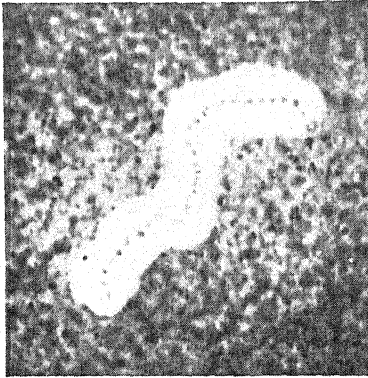


Fig. 10-4. Streptococci, showing well developed capsules. Background is India ink ( $\times 1500$ ). The use of India ink is a form of negative staining. (Courtesy Dr. Sidney Rothbard. J. Exp. Med., 1948, vol. 88.)

are of great importance in many aspects of microbiology as we shall see later on, especially in medical microbiology.

THE RELATION OF CAPSULE TO CELL is not always clear. In some species capsules seem to be a thickened layer of the cell wall; in others, a secreted, mucoid coating (Fig. 10-4). Some bacteria secrete much slimy substance into their environment. This may not be a true capsule and may bear no more true structural relation to the cell proper than any excreted waste product. Some bacteria produce slime in addition to capsules.

In many species large, easily seen capsules seem to be called forth only by certain particular circumstances of environment such as growth in the animal body, or on certain media. Some, for example *Leuconostoc*, form their capsular material best in sugar vats or in milk. There is good reason to believe also that capsule formation may be stimulated, in some species, by unfavorable environments such as the resistance of an infected host. For example, capsules are often found well developed on, and demonstrably correlated with, virulence of pathogenic forms such as pneumococci and the organisms of whooping cough, gonorrhea, meningitis, anthrax and other diseases. The capsules seem to enable these bacteria to evade the defensive mechanisms of the infected body. The bacteria often cease forming capsules when removed from the body to laboratory test-tube media.

All capsules, whether occurring on pathogens or saprophytes, may well be regarded as a form of protective coating produced by the organisms in response to unfavorable external conditions or other stimulus.

✓ COMPOSITION OF CAPSULES. The chemical composition of capsules varies widely with species. Most capsules are composed mainly of carbohydrates or gums; others also contain nitrogen and phosphorus and may be of the nature of mucus or the mucins. The capsule of some species appears to consist of fibrous polypeptide micellae. In others the capsule may be composed of curiously cross-striated fibers similar to the collagen of human connective tissue.

Capsular substance often has *specific chemical properties* which enable us to distinguish between various closely similar types of organisms which *could not otherwise be differentiated*. This is an important point to remember. The methods used will be made clear later.

DEMONSTRATION OF CAPSULES. There are several methods of demonstrat-

ing the presence of capsules on bacteria. As capsules of various species differ in composition, however, they are not all amenable to the same process. A method useful for staining many capsules is given below.\*

Capsules, like flagella, do not have a marked affinity for basic dyes and some capsules are easily removed by mechanical means or by washing with water.

**3. Cell Wall.** As with most bacterial structures it is not safe to generalize about the structure and function of the cell wall. What is true of one species may be quite different in another. By cell wall is meant (for bacteria) the thin, sharply-defined, relatively tough (though flexible), somewhat rigid structure just inside the slime layer or capsule. The cell wall is generally physically and chemically distinct from the capsule and from the inner cytoplasmic membrane. It is estimated to range in thickness around  $0.02\mu$ . Rough analogies of a cell wall would be the casing of an automobile tire (not the tubeless type) or the leather covering of a basketball.

The cell wall in some microorganisms may be beautifully shown by special staining procedures and by the electron microscope. Methods of differentiating the cell wall and cytoplasmic membrane have been described. Both stain with difficulty and generally require a mordant such as tannic acid or phosphomolybdic acid.

The cell wall varies in thickness, rigidity, strength and chemical composition with species and growth conditions and other unknown factors. A good idea of the character of some bacterial cell walls is obtained with the electron microscope, examining ruptured bacteria and also very thin slices of bacteria embedded in material like wax or soft plastic (Fig. 10-5).

✓ THE CHEMISTRY OF THE CELL WALL is not well established. In general bacterial cell walls appear to be rather stable and resistant to the action of most substances except strong acids and alkalies. In some fungi the cell wall appears to be mainly chitin. Most studies of bacteria indicate the presence of carbohydrates, or carbohydrate complexes, chitin or chitin-like compounds, mucinoids, hemicellulose and similar substances, though species differ greatly. Cellulose is not common. In gram-positive bacteria the cell wall is thought to contain the complex (ribonucleic acid?) responsible for the gram-positive reaction.

\* Capsule stain [Welch method (modified)]:

A. Crystal violet

Saturated alcoholic crystal violet..... 5 ml

Distilled water..... 95 ml

B. 10 per cent  $\text{CuSO}_4$ ..... 1 liter

1. Make thin smear.

2. Dry in air.

3. Fix by gentle heat.

4. Cover with crystal violet solution.

5. Heat to steaming for 1 second. (Do not boil!)

6. Use no water.

7. Rinse immediately and copiously with copper sulfate solution.

8. Use no water.

9. Blot dry immediately.

10. Examine.

The organisms are stained purple and the capsule appears as a faintly blue halo. See also other methods referred to.

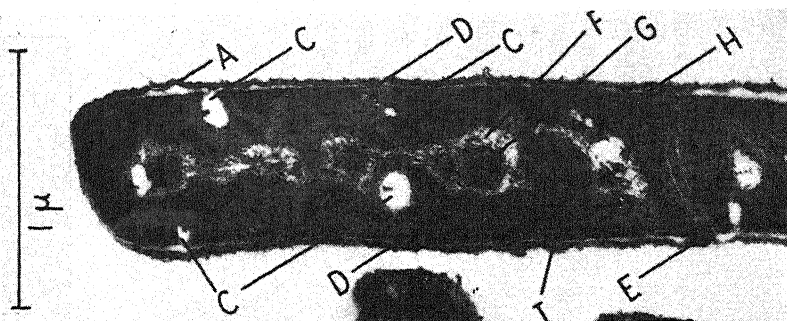


Fig. 10-5. *Bacillus cereus*, a common, harmless bacterium found in dust everywhere has been cut lengthwise in a very thin section (see scale of  $1\ \mu$  at left). This is an electronograph at a magnification, originally, of  $\times 60,000$ . The section shows two adjacent cells and beginning formation of a third. A, cell wall showing evidence of the shrinking of the cytoplasm. C, indicating four peripheral bodies cut at different levels. D, beginning of a centripetally growing transverse cell wall. E, completed transverse cell wall before thickening. F, low-density fibrous component of supposed nuclear apparatus. G, dense body in nuclear apparatus which may be inclusion of cytoplasmic material. H, small dense particles which appear to be main constituent of cytoplasm. I, unidentified cytoplasmic inclusions. (From the collection of the Society of American Bacteriologists, courtesy of Dr. G. B. Champan, RCA Laboratories Division, Princeton, N. J.)

THE STRUCTURE OF THE CELL WALL is sometimes thought of as open, like the ribs of a ship. This would indicate that the cell wall is chiefly an inert supporting structure. Others regard it as more continuous; a mosaic of various molecular complexes. Probably both structures occur.

Whatever the makeup of the cell wall it is, in all plant cells (including bacteria), one of the structures through which must pass inward not only the nutrient material for the cell, but outward all waste matter, digestive enzymes and other products given off by the cell during its life. In order to help retain within the cell the proper substances in the proper proportions, and to prevent the entrance and allow the escape of improper or waste substances, it must exert a very fine discrimination or selective permeability at all times. In this it is doubtlessly aided by the cytoplasmic membrane.

Any slight defect or alteration in the discriminatory properties of the cell wall must, therefore, have a profound effect on the cell contents. When, as is the case with bacteria, the entire individual consists of the cell contents and depends for its existence as a living entity on the chemical and physical equilibrium of its protoplasm, then the maintenance of the integrity and uniformity of the cell wall is a matter of the greatest moment. Substances or forces which tend to alter the wall or overcome its selective powers must influence the well-being of the cell. These will be discussed later on.

**4. Protoplasm.** The protoplasm comprises all of the material inside the cell wall. The protoplasm appears to have, at its outer surface, a thin, enclosing membrane: the protoplasm membrane. This is equivalent to what is often called the cytoplasmic membrane. The protoplasm content is equivalent to cytoplasm plus nuclear structures plus other inner, essentially cellular materials. The protoplasm is the "living stuff" of the cell; i.e., it is protoplasm (see Fig. 10-12).

The intact protoplasm is liberated when the cell wall is dissolved. This may

be accomplished, in one way, by means of the enzyme *lysozyme*.\* The protoplasts of bacilli assume a spherical shape after the cell wall is dissolved, showing that the cell wall of such organisms is a rigid, supporting structure without which the protoplast is a limp, protoplasmic mass. Flagella of motile bacilli are not removed with the cell wall but normally protrude through it, being rooted in the cytoplasm.

The protoplast membrane appears to have closely associated with it certain enzyme systems which mediate respiratory functions involving oxygen at the cell surface. These are discussed further in Chapter 13.

Freed from the cell wall the protoplast appears to be able to carry on all of the essential functions of the complete cell, including growth and fission, with only slight modifications. For example, it cannot effectively move its flagella. Its osmotic properties also appear to be altered since free protoplasts tend to lose water. The most obvious functions of the cell wall are thus revealed as: (a) accessory to flagellar motion, possibly in the role of "oar-locks" for flagella; (b) protection of the protoplast; (c) maintenance of the elongated form of bacilli; for reasons given elsewhere this seems to be a physiologically more efficient form than the spherical; (d) supplementation of the osmotic properties of the protoplast membrane.

(a) PROTOPLAST MEMBRANE. When, in plasmolysis, the bacterial cytoplasm shrinks from the cell wall, it appears to be enclosed in an exceedingly thin, but distinct, membrane (*the protoplast membrane*). This is normally in intimate contact with the inside of the cell wall like the inner tube of a tire. The chemical nature of this cytoplasmic membrane is not clear. It may consist of a condensation or slight thickening of certain surface-active elements of cytoplasm where it is in contact with the cell wall. The remarks on the importance of selective permeability and alterations in structure made in connection with the cell wall also apply to the cytoplasmic membrane. The two are undoubtedly of fundamental import in acting as mediators between the living cytoplasm within, and the world outside.

(b) CYTOPLASM. This is one part of the protoplast of all typical cells. The cytoplasm is usually clear, watery or slightly viscous, and homogeneous in appearance although granules of various substances, such as stored food, oil, etc., may be suspended in it. It consists of a complex mixture including proteins, lipids, carbohydrates, minerals and water. The organic matter is largely in the colloidal state, and so intimately intermingled as to defy synthesis and any analysis but the grossest sort. In larger cells than bacteria the fluid part of protoplasm is in constant circulatory motion called *protoplasmic streaming*. This has not been observed in bacteria though it may occur.

(c) NUCLEAR STRUCTURES. It is generally thought that something in bacterial cells must act like a nucleus in order to carry on and control such functions as various chemical activities, reproduction, heredity, and life itself. However, neither the nature nor the existence of the nucleus of bacteria is certain.

A chief difficulty is the minute size of bacteria. A nucleus, if it exists, would be barely within the limits of visibility of the best of light microscopes. Through the combined use of electron and light microscopy, and certain

\* Lysozyme was discovered in 1922 by Fleming who later discovered penicillin. Lysozyme occurs in egg-white, various bodily secretions and may be produced by certain bacteria.

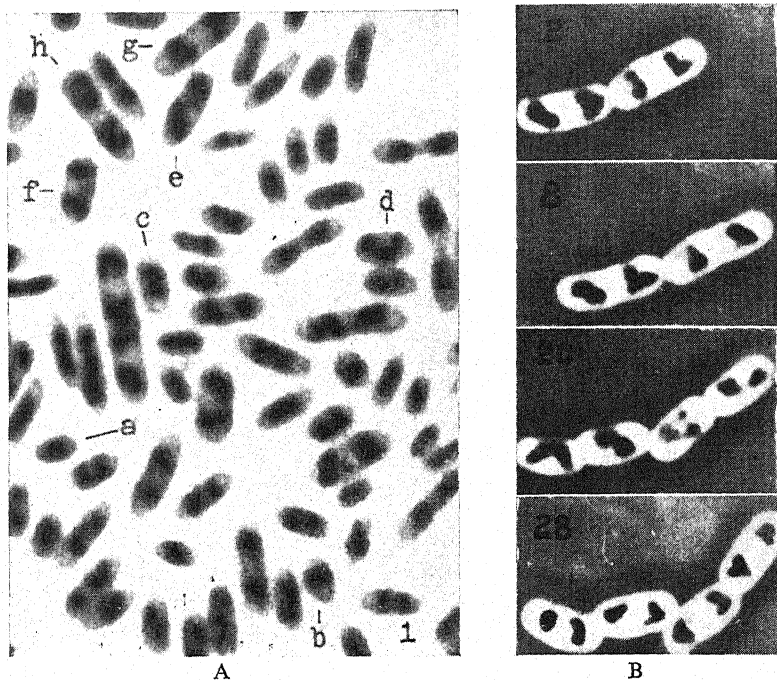


Fig. 10-6. *A, a-h* are interpreted as successive stages in the development of a coccoid cell with a broad (double?), central, chromatinic structure into a typical rod-shaped two-cell bacterium with two pairs of chromosomes. Note the V-shaped division stage at *d*. The chromosome forming the left limb of the V is so much broader than the right one that it seems plausible to assume that it is preparing to divide. At *e* a thin strand of chromatinic matter is seen connecting recently divided chromosomes. A difficulty lies in not knowing the order of events; for example, whether *a* normally precedes *b* or vice versa. Since each is a different cell and all were photographed simultaneously one has to assume the order of development. (Robinow, C. F.) *B*, these difficulties are eliminated to some extent by continuous observation of single cells. In *B* are seen nucleus-like bodies in living, multiplying bacteria (*Escherichia coli*) photographed microscopically at intervals, a difficult procedure. The numbers in the pictures indicate age in minutes of the cells from the moment of first observation. No evidence of mitotic figures was observed and no pattern of nuclear division could be formulated from such series of observations. (*B*, courtesy of Drs. D. J. Mason and D. M. Powelson, in *J. Bact.*, 1956, vol. 71.)

chemical staining methods, a number of clever scientists have revealed granules and remarkable internal changes in bacteria which suggest nuclear structures and activities (Figs. 10-6 and 7). Doubt persists because of the difficulty in proving their nuclear nature unequivocally.

The principal views on bacterial nuclei (except for those denying the presence of any nucleus) may be summarized as follows:

1. *There is a true nucleus and cytoplasm.* This has been discussed above.
2. *There is a true nucleus but no cytoplasm.* When ordinary stains are used, the bacterial cell as a whole stains like nuclear material and no differentiation between nucleus and cytoplasm can be made with the microscope. When special stains are used, differentiations of the cell contents may be seen but their exact nature is not known with certainty.

3. The nucleus consists of minute particles of nuclear material, or granules of chromatinic material, diffusely scattered in the cytoplasm, perhaps attached to a mitochondrial reticular network of threads; or perhaps strung on a long mitochondrial thread, the whole resembling a tangled string of beads enclosed within the cytoplasmic membrane. This is a widely held view at present. In order to explain the appearance, in electronographs, of fairly large, discrete bodies said to be nuclei or analogous structures, it has sometimes been suggested that the scattered nuclear granules may coalesce into functional nuclei just before cell fission. This is a not unreasonable view and is consistent with many observations.

While it must be admitted that we do not know very much about the morphology of bacterial nuclei, we cannot deny that something carries on the functions of a nucleus in a fairly efficient manner.

**Mitochondria-like Particles.** In some bacteria, protein particles of definite size, distinctive specific gravity, and demonstrable enzymatic activity have been differentiated in the cytoplasm. The exact nature of these is not clear but they appear to be, or to act as though they contain, enzymes. Some have called them *mitochondria*, since they appear to resemble these cytoplasmic granules which occur in the cells of animal tissue.

**Spores.** Only three genera of true bacteria (*Bacillus*, *Clostridium* and *Sporovibrio*) form spores.

When a rod of the genus *Bacillus* sporulates, an area of altered cytoplasm appears in the cell. This undergoes modifications and develops an outer wall. The exact nature of the process is not clear. Some data indicate that spores develop from a single primordium of nuclear material; others that it includes nuclear granules. It has been suggested that spore formation is accompanied by sexual functions, such as automixis, or nuclear fission or fusion. Whether this is so or not, part or all of the vital cell content is finally condensed and

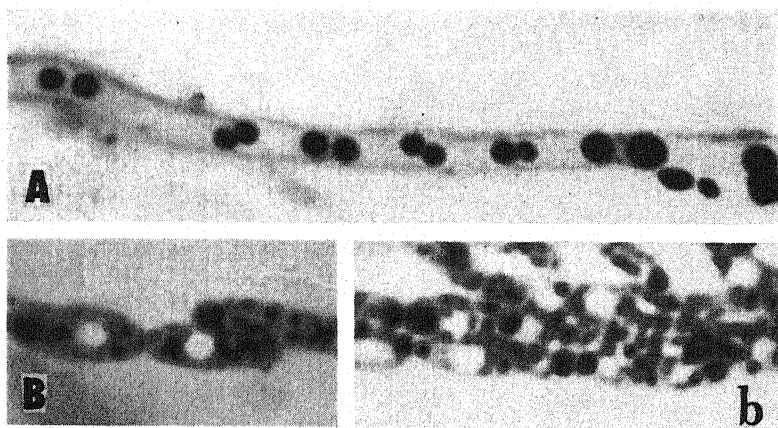


Fig. 10-7. Structures interpreted as bacterial nuclei in a common species of *Bacillus* (*B. cereus*). In *A* note division of nuclei in association with division of the cells. These are noted as paired, compound, probably sister nuclei. In *B* and *b* note the internal structures of compound nuclei which develop from the nuclei shown in *A*. (Photos courtesy of Dr. Georges Knaysi, in *J. Bact.*, 1955, vol. 69.) These are from electronographs; each cell is about  $5\mu$  in length.

surrounded by a relatively thick shell. The material in the spore appears to be packed inside the spore membrane in a very orderly manner, like the germ in a seed. It probably consists largely of nuclear material or DNA (see Fig. 33-2).

In the genera of bacteria named above one spore is formed inside each cell, and each spore is properly called an *endospore*. Some spore-forming species are also said to occur in the gram-negative genera *Vibrio* and *Spirillum* and the gram-positive *Sarcina*.

**PARASPORAL BODY.** During spore formation by some (possibly all) spore-forming, aerobic bacilli a small granule, called a *parasporal body*, appears. It is not inside the spore proper, but is closely associated with it and seems to be attached to the outer surface. It is spheroid and, in diameter, about  $\frac{1}{5}$  of the length of the spore. Its significance is unknown.

The state of the water in the spore is not certain. There is evidence that the spore is dehydrated. Contrary data indicate that the water content is unchanged but that the water in the spore is not in a free state, being *bound*\* or imbibed by the lyophilic colloids in the spore. Dehydration seems more probable. Either, as well as an increased calcium content of spores, would seem to account for the heat resistance of endospores.

The cell with fully formed spore is called a *sporangium*. The empty cell wall of the bacillus may separate off, like the hull of a seed, leaving the spore as a free, round, or oval body. Disinfectants cannot readily penetrate into spores, nor can aniline dyes.

**STAINING SPORES.** A rod with a spore inside, when stained by ordinary methods may thus appear to have a hole in it (Fig. 10-8). However, the outer shell of the spore may readily be colored so that, in stained smears, free spores may appear as tiny, blue or red rings. There are special stains for spores.†

**SPORE FORMATION.** Endospores are formed most readily under good growth conditions. Their formation appears to require some energy food such as glucose. In some species manganese ions are requisite. In others iron and manganese greatly increase spore formation. Apparently at least part of the spore contents are newly synthesized and are not merely old material of the vegetative cell. Certain specific nitrogenous food substances (as certain amino acids) are required. The fact that by far the largest numbers of spores are usually found in well-matured cultures several days old indicates that spores generally represent a mature stage of cell development.

There is a curious difference between aerobic (*Bacillus*) and anaerobic (*Clostridium*) sporulation. The aerobic bacilli do not sporulate under strictly anaerobic conditions; the anaerobic bacilli do not sporulate under aerobic conditions.

**GERMINATION OF SPORES.** When spores are placed in nutrient fluids at favorable temperatures the spore sac splits and a vegetative, active, bacterial cell emerges (Fig. 10-9). *Germination* is said to have occurred. The method of splitting is thought to be distinctive of species. Probably water is taken up.

\* The apparent dryness of a hard-boiled egg is a good illustration of bound water.

† **Spore stain:** Apply saturated malachite green (about 7.6%) for 10 minutes.

Flame until warm.

Rinse with tap water for about 10 seconds.

Apply 0.25% aqueous safranin for 15 seconds.

Rinse with tap water, blot, and dry.

Certain food substances favor germination: manganese ions, *l*-alanine, and other amino acids, glucose, etc.

Spores of anaerobes will *germinate* readily under *aerobic* conditions and aerobic spores will *germinate* under *anaerobic* conditions.

*Dormant spores* show little metabolic activity, but they *are* alive and do show a low rate of respiration.

**FUNCTION OF SPORES.** Sporulation of bacteria obviously is not a necessary function since most species reproduce perfectly without demonstrable sporulation. Further, in bacteria, it is generally thought, only one spore is produced by each cell and this spore produces only one cell; i.e., spore formation is not a reproductive mechanism. In this respect bacterial spores differ from the spores of true fungi and higher plants. Bacterial spores appear to represent merely a dormant and resistant state which enables the bacteria producing them to survive during periods unfavorable to active vegetation. However, some workers maintain that more than one spore is formed per cell. The question is in debate.

**RESISTANCE OF SPORES.** When protein or protoplasm is moist or well hydrated, as is its condition in an actively growing, vegetative cell it coagulates readily and is therefore vulnerable with respect to heat, chemicals, etc. The "white" of fresh egg is a good illustration of a hydrated protein. It coagulates ("hard boils") readily enough.

When protein is dehydrated, or when the protein and water are bound, it

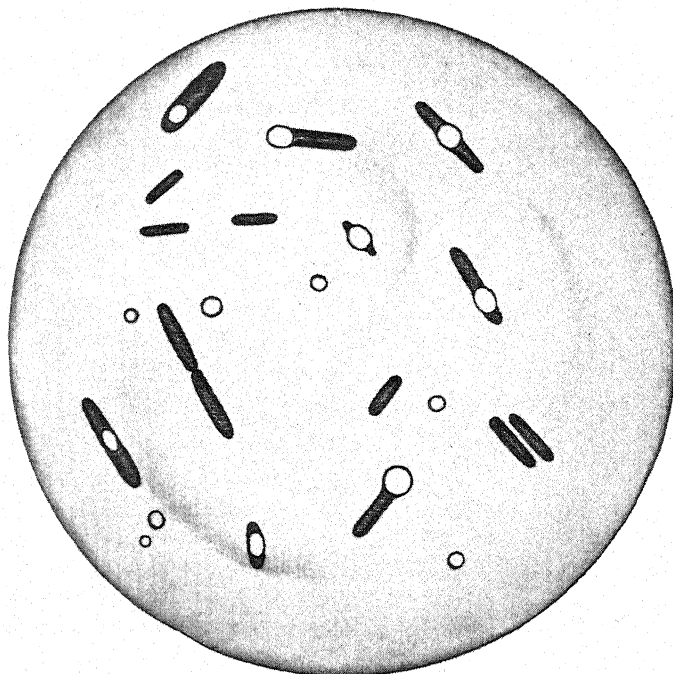


Fig. 10-8. Various types of bacterial spores. Some of the spores have escaped from the sporangia. Stained with methylene blue, which does not penetrate inside the spore, only the outer surface of the spore is stained.





Fig. 10-9. Electronograph of *B. mycoides* showing rupture and germination of a spore. The "cap" of the spore wall is seen at the left, while the bacillus arises out of the remainder of the spore shell below. (Courtesy of Knaysi, Baker and Hillier, *J. Bact.*, vol. 53.)

is no longer readily coagulable. It is then attacked only with difficulty by various chemicals, and withstands high temperatures and other conditions which would soon destroy it were it allowed to become hydrated. Egg powder is a good example of dehydrated protein.

Partly because of their apparently dehydrated state spores can survive where the active, vegetative, or hydrated stage would soon perish. So resistant are some bacterial spores that they will live for decades, and probably for centuries, on dry splinters of wood, grow after treatment in strong disinfectant solutions, survive an hour or more of boiling, or even of pressure-cooking (autoclaving) at 120° C, and remain unharmed by an hour or more in a hot oven. Not all spores are so resistant, however, some being killed at temperatures little above those lethal to vegetative cells (60° C–70° C). However, most bacterial spores are quite resistant.

It is sometimes difficult or impossible to demonstrate spores by means of the microscope, even when staining methods are employed. Resort must then be had to tests for resistance of the culture to heat. A culture which resists 85° C for 20 minutes almost certainly contains spores although they may not be visible.

There is some evidence that high growth temperatures of the spore-forming bacilli enhance the resistance of their spores to heat. For example, thermophilic species (those growing only at high temperatures (60° C–75° C) usually produce the more heat-resistant spores.

**Granules.** In many bacteria stained by ordinary methods, granules are readily seen. They are distinguished by the fact that they stain more intensely than the rest of the cell, or are of a different color. In this case they are often spoken of as *metachromatic* granules. We have already mentioned a number of kinds of granules, some resembling mitochondria. The composition and function of some types of granules is a matter of debate. As shown by their reaction to reagents such as iodine, some of them are of *starch* (blue), *gly-*

*cogen* (brown) or closely related compounds and doubtless often represent reserve food substances since they tend to disappear during periods of food scarcity. In some species globules of pure elemental *sulfur* are stored as food in the cell.

**VOLUTIN.** In many bacteria, granules of *volutin* are commonly observed (Fig. 10-10). These have often been confused with nuclei. Volutin is a food material, probably consisting of inorganic polyphosphates and nucleic acid-like compounds. These are not identical with the protein of the nucleus, as they are dissolved in hot water (80° C) instead of being coagulated by it, and are not digested by trypsin.

**FAT.** Some bacteria synthesize and store much *fat*, especially if fed with sugar or other assimilable food; in this showing a striking resemblance to many human beings. Some species of the genus *Bacillus* are outstanding in this respect, but other bacteria, yeasts and fungi also store up much fat (see Fig. 10-11). A 24-hour culture on glycerol agar, if properly stained, will show large amounts of fat.\* The method offers a good field for investigation of problems in metabolism morphology, taxonomy, etc.

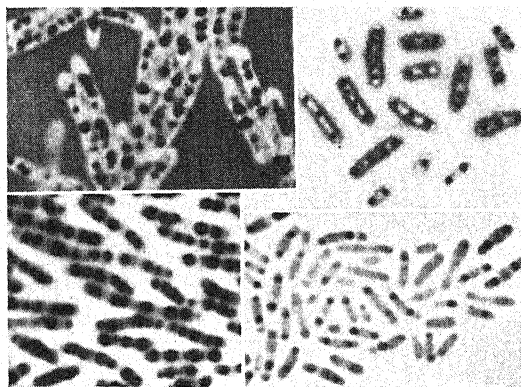


Fig. 10-10. A common, harmless bacterium (*Aerobacter aerogenes*) stained by the Albert-Laybourn method for demonstrating volutin. It is interesting to note the striking similarity of these granules to the bodies interpreted as nuclei by some workers. Magnification originally at  $\times 3000$ . (Courtesy of Drs. J. P. Duguid, I. W. Smith and J. F. Wilkinson, Dep't of Bacteriology, University of Edinburgh, Scotland. In *J. Path. and Bact.*, 1954, vol. 67; also *J. Bact.*, 1954, vol. 68.)

\* **Fat stain (Burdon)**

1. Prepare the film, dry and fix by gentle heating as for the Gram stain.
2. Flood the entire slide with Sudan Black (0.3 per cent dry dye in 70 per cent  $C_2H_5OH$ ) and leave the slide for 5 to 15 minutes. Drying of the dye will not harm the preparation. Use of a Coplin jar is less satisfactory.
3. Drain off excess dye and blot dry.
4. Clear in xylol for about 1 minute, dipping in and out.
5. Counterstain with 0.1 per cent aqueous safranin for 10 seconds. Overstaining with counterstain will mask the fat stain. It may even be omitted.

The fat appears as dark, blue-black masses; the rest of the cell is pink.

The stock of solution of Sudan Black keeps well in a clean, stoppered bottle. It should be occasionally shaken and allowed to settle overnight before use. Sudan III and Scharlach R are *much less* satisfactory.

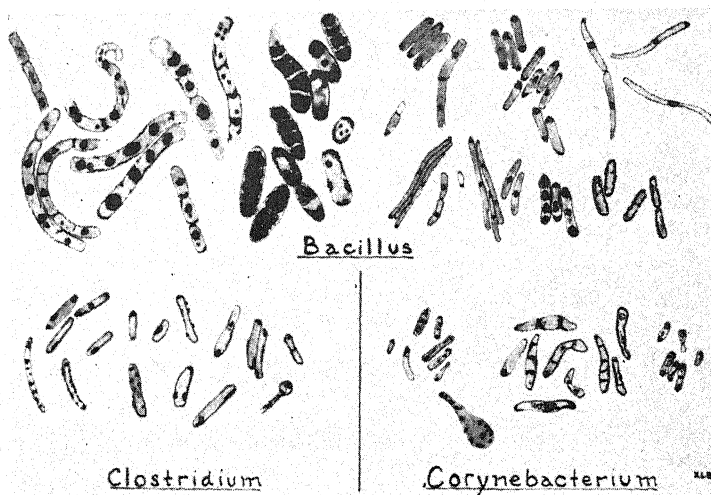


Fig. 10-11. Various species of bacteria stained for fat with Sudan black. Note the similarity of many of these masses to pictures representing what is said to be nuclear material. (Burdon, K. L., in J. of Bact., vol. 52.)

**Staining Granules.** There are many methods of bringing granules into prominence, the method depending largely on the chemical nature of the granules themselves. One commonly used to demonstrate volutin granules is that of Albert.\*

✓ **Chemical Composition of Bacteria.** Bacteria are not markedly different in chemical composition from other living cells. However, the *exact* chemical analysis of *living* matter is difficult, if not impossible, since to analyze it one must kill it and disturb the interrelations of various components of the cell.

Bacterial *protoplasm* is like all other protoplasm in that it is composed chiefly of the elements C, O, H, S, N and P, with smaller amounts of many other elements as Na, K, Ca, Fe, Mg, Cl and so on. Species differ in the actual amounts of each element present. The actual proportion of each of these elements usually varies greatly with the composition of the medium on which the bacteria are growing at the time they are analyzed.

In vegetative bacterial cells, water is an important component, constituting, roughly, from 75 to 98 per cent of the whole weight. The proportion is quite variable, depending on environment.

\* **Albert's method of staining volutin granules**

A. Toluidin blue.....	0.15 gm
Methyl green.....	0.20 gm
Acetic acid (glacial).....	1.00 ml
Alcohol (95 per cent).....	2.00 ml
Water.....	100.00 ml

Allow to stand 24 hours. Filter through paper.

B. Iodine as in Gram's stain.

1. Prepare smear as usual.

2. Flood with solution A for 1 minute. Wash with water and blot dry.

3. Stain with solution B for 1 minute, wash and blot dry.

Granules black; other parts dark or light green.

Growth in saline media increases ash content. The ash content (inorganic matter) may vary in amount from 2 to 30 per cent of the *dry* weight of bacteria; organic matter from 40 to 90 per cent.

The organic matter consists of:

- |   |       |
|---|-------|
|   | %     |
| 1. Proteins (including nucleoproteins).....                             | 40-80 |
| 2. Carbohydrates*.....  | 10-30 |
| 3. Lipids.....  | 1-30  |
| 4. Nucleic acids (RNA and DNA†).....                                    | 5-30  |
| 5. Miscellaneous organic compounds occur in lesser and varying amounts. |       |

The cell walls contain chitin or chitin-like compounds in some species, carbohydrate complexes in others. Chitin was formerly regarded as distinctively an animal substance.

Many complex, non-protein, nitrogenous substances are also found, among them various purine bodies, polypeptides, and amino acids. All but two of the known amino acids have been found.

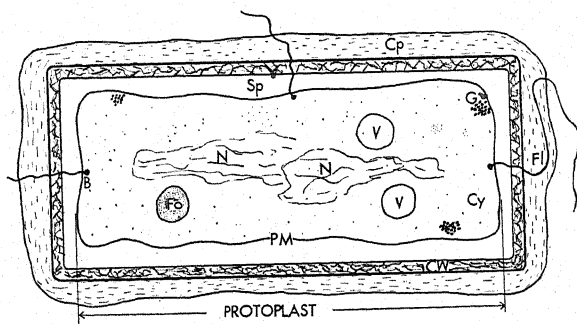


Fig. 10-12. Structures commonly seen in cells of representative† species of rod-shaped bacteria. For discussion see text. N = nuclear material; form and distribution indeterminate. Cy = cytoplasm. G = dense, intracellular granules, probably containing RNA; often closely associated with enzyme activity. V = vacuole-like structures, possibly emptied of lipid, volutin or other material during preparation of the specimen for microscopy. Fo = granules of volutin, fat, polysaccharide or other stored food substances. CW = cell wall. Fl = flagellum. B = basal granule or blepharoplast-like granule associated with attachment and/or function of flagella. Cp = capsule. PM = protoplast membrane. The space (Sp) between protoplast membrane and cell wall is ordinarily non-existent. It has been exaggerated here for diagrammatic purposes. An appearance such as that in the diagram might occur as a result of plasmolysis. Note also that, while flagella and well developed capsule are here shown together for diagrammatic purposes, they do not commonly occur together on the same cell.

\* Cultivation in carbohydrate-rich media increases carbohydrate and lipid content. Curiously, glycogen is found among the list of carbohydrates. Glycogen was formerly regarded as a distinctively animal substance.

† DNA was for some time thought of as a distinctively animal substance. Nucleoproteins often constitute up to 80% of the total organic matter in the cell. DNA is more to be associated with the nucleus, RNA with cytoplasm.

‡ This is hypothetical because of the wide differences between the various orders of bacteria. However, it is reasonably typical and serves as a useful basis for elementary discussion.

The general structure of nucleoproteins and nucleic acids may be seen in Figure 5-6.

**Pigments of Microorganisms.** Colonies of most bacteria growing on dye-free media are greyish, whitish and translucent or opaque. However, there are some notable exceptions. Pigments are a prominent feature in some species. In most of these their exact function is not known nor is their chemistry, except for photosynthetic pigments of the few alga-like species. In color they range the spectrum.

Some pigments may be excreted as colorless compounds called leukobases, becoming colored only on oxidation after excretion. A good example is the blue *pyocyanin*, a phenazine derivative produced by a common saprophyte (sometimes pathogen) *Pseudomonas aeruginosa*.

Many of the pigments are of the group of red, yellow or orange *lipochrome compounds*, common in flowers, butter, egg yolks, corn, etc. *Cytochrome*, the respiratory pigment of aerobic bacteria, contributes no visible coloration to them. Bacteriochlorophyll gives a purple or greenish color to the alga-like photosynthetic species (Rhodobacteriaceae). These are discussed in Chapter 29.

Pigments in general vary greatly in amount and intensity of color, even in the same organism. Growth at various temperatures and on various media affects either the mechanism responsible for pigment formation or the color of the pigment itself. Pigments usually develop best on solid media in close association with air. Anaerobic growth usually results in lack of, or discoloration of, bacterial pigment. Pigmented species may give off non-pigmented variants. Pigments may be intracellular, or they may pass through the membrane of the cell and so be extracellular.

#### REFERENCES

- Alexander, M.: Localization of enzymes in the bacterial cell. *Bact. Rev.*, 1956, 20:67.  
 Bartholomew, J. W., and Clare, P.: Parasporal bodies of bacillus spores. *J. Bact.*, 1956, 71:158.  
 Bartholomew, J. W., and Mittwer, T.: Simplified bacterial spore stain. *Stain Technol.*, 1950, 25:153.  
 Bissett, K. A.: *The Cytology and Life History of Bacteria*. The Williams & Wilkins Co., Baltimore, Md., 1955.  
 Bradfield, J. R. G.: Electron microscope observations on bacterial nuclei. *Nature*, 1954, 173:184.  
 Burdon, K. L.: Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *J. Bact.*, 1946, 52:665.  
 Chance, H. L.: A study of bacterial substances stainable by the hydrogen chloride method. *J. Bact.*, 1956, 71:285.  
 Chapman, G. B.: Electron microscopy of ultra-thin sections of bacteria. II. *J. Bact.*, 1956, 71:348.  
 Church, B. D., Halvorson, H., and Halvorson, H. O.: Studies on spore germination: its independence from alanine racemase activity. *J. Bact.*, 1954, 68:393.  
 Clark, J. B., and Webb, R. B.: Ploidy studies on the large cells of *Micrococcus aureus*. *J. Bact.*, 1955, 70:454.  
 DeLamater, E. D.: A conceptual formulation of the structure and divisional mechanism of the bacterial nucleus. *Tr. New York Acad. Sci.*, 1954, Ser. II, 16:366.  
 DeLamater, E. D.: The bacterial nucleus. *Ann. Rev. Micr.*, 1954, 8:23.  
 Dondero, N. C., Adler, H. I., and Zelle, M. R.: Quantification of the Feulgen reaction in bacteria. *J. Bact.*, 1954, 68:483.  
 Duguid, J. P.: The demonstration of bacterial capsules and slime. *J. Path. and Bact.*, 1951, 63:673.

- El-Bisi, H. M., and Ordal, Z. J.: The effect of certain sporulation conditions on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. *J. Bact.*, 1956, 71:1; see also p. 11.
- Foster, J. W., and Perry, J. J.: Intracellular events occurring during endotrophic sporulation in *Bacillus mycoides*. *J. Bact.*, 1954, 67:295.
- Hachisuka, Y., et al.: Studies on spore germination. *J. Bact.*, 1955, 69:399.
- Henrici, A. T.: Morphologic Variation and the Rate of Growth of Bacteria. Charles C Thomas, Springfield, Ill., 1928.
- Hewitt, L. F.: Effect of cultural conditions on bacterial cytology. *J. Gen. Micro.*, 1951, 5:293.
- Hoffman, H.: The cytochemistry of bacterial nuclear structures. *J. Bact.*, 1951, 62:561.
- Knaysi, G.: Cytology of bacteria. *Ann. Rev. Microbiol.*, 1956, 10:253.
- Knaysi, G.: The structure, composition, and behavior of the nucleus in *Bacillus cereus*. *J. Bact.*, 1955, 69:117.
- Lamanna, C., and Mallette, M. F.: Basic Bacteriology. Williams & Wilkins Co., Baltimore, Md., 1953.
- Leifson, E., Carhart, S. R., and Fulton, M.: Morphological characteristics of flagella of *Proteus* and related bacteria. *J. Bact.*, 1955, 69:73.
- Leifson, E., and Plaen, M. I.: Variations and spontaneous mutations in the genus *Listeria* in respect to flagellation and motility. *J. Bact.*, 1955, 70:233.
- Lessler, M. A.: The nature and specificity of the Feulgen nuclear reaction. *Internat. Rev. of Cytol.*, 1953, 2.
- Levinson, H. S., and Hyatt, M. T.: The stimulation of germination and respiration of *B. megaterium* spores by manganese, l-alanine and heat. *J. Bact.*, 1955, 70:368.
- Mason, D. J., and Powelson, D. M.: Nuclear division as observed in live bacteria by a new technique. *J. Bact.*, 1956, 71:474.
- Mitchell, P., and Moyle, J.: Liberation and osmotic properties of the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea*. *J. Gen. Micro.*, 1956, 15:512.
- Morowitz, H. J.: The energy requirements for bacterial motility. *Science*, 1954, 119:286.
- Mudd, S.: The bacterial cell. *Ann. Rev. Micro.*, 1954, 8:1.
- Oginsky, E. L., and Umbreit, W. W.: An Introduction to Bacterial Physiology. W. H. Freeman and Co., San Francisco, 1954.
- Peterson, R. G., and Hartsell, S. E.: The lysozyme spectrum of the gram-negative bacteria. *J. Inf. Dis.*, 1955, 96:75.
- Pijper, O., and Nester, M. L.: The wavelengths of helical bacterial flagella. *J. Gen. Micro.*, 1956, 14:371.
- Roth, N. G., and Lively, D. H.: Germination of certain aerobic bacilli under anaerobic conditions. *J. Bact.*, 1956, 71:162.
- Schmidt, C. F.: The resistance of bacterial spores with reference to spore germination and its inhibition. *Ann. Rev. Micro.*, 1955, 9:387.
- Sneed, T., and Halvorson, H. O.: The fats of *Aerobacter cloacae*. *Appl. Micro.*, 1954, 2:285.
- Spooner, E. T. C., and Stocker, B. A. D., Editors: Bacterial Anatomy. 6th Sympos., Soc. Gen. Micro., 1956. Cambridge Univ. Press, New York.
- Stedman, Ed., and Stedman, El.: The cytological interpretation of the Feulgen reaction. *Biochem. J.*, 1950, 47:508.
- Waldham, D. G., and Halvorson, H. O.: Studies on the relationship between equilibrium vapor pressure and moisture content of bacterial endospores. *Appl. Micro.*, 1954, 2:333.
- Weinberg, E. D.: The effect of  $Mn^{++}$  and antimicrobial drugs on sporulation of *Bacillus subtilis* in nutrient broth. *J. Bact.*, 1955, 70:289.
- Werkman, C. H., and Wilson, P. W.: Bacterial Physiology. Academic Press, New York, N. Y., 1951.
- Widmer, C., King, T. E., and Cheldelin, V. H.: Particulate oxidase systems in *Acetobacter suboxidans*. *J. Bact.*, 1956, 71:737.
- Widra, A.: Studies on the cytochemistry of bacteria. *J. Bact.*, 1956, 71:689.

## Classification and Taxonomy of Bacteria

WHILE ALL agree that classification and taxonomy are desirable and necessary phases of biological work there is much disagreement as to the best method of classifying and naming living things. The zoologists and the botanists have arranged matters in their fields fairly satisfactorily, largely on the basis of morphological characters. These are a good guide to *natural* or *evolutionary* relationships because they are based on a long, connected and demonstrable history of evolutionary development and phylogenetic relationships. The microbiologist can only speculate as to the evolutionary and phylogenetic history of microorganisms; virtually nothing is known of them. Hence he has not arrived at systems of classification in which the natural relations of the organisms are used as a basis.

**Development of Bacterial Classification.** Nevertheless, he has systems of classification. They have been built up largely as arbitrary *descriptive keys for identification* of organisms according to whatever information about them may be available. These descriptive keys cannot properly be called natural classification. They are indispensable, but their artificial and entirely arbitrary nature must not be forgotten. They have no bearing on natural or evolutionary relationships of microorganisms.

Organisms may be arranged in systematic groups, called *taxa*, according to shape, motility, or other criteria. The science and art of naming such groups is called *taxonomy*. Form, motility, spore formation, growth under certain absolute conditions such as complete lack of oxygen (anaerobiosis), and similar properties are qualitative, *unit characters*. They are most useful in classification because they are not relative and variable like size or rate of growth. They are present or not present.

**NATURE OF DIFFICULTIES.** In dealing with recently discovered organisms, having *clearly described* properties which can be readily determined by generally available methods; and of which sample cultures are available for other workers to study, classification may not be too difficult a task. If, however, the person originally naming a supposedly newly discovered organism failed to keep cultures available for study, used undescribed or misleading methods to determine color, motility, biochemical properties, etc., and if, in addition, he was unacquainted with the rules of nomenclature (rules governing the naming of microorganisms) and used incorrect names or names already given to other organisms, it can be seen that this worker has introduced confusion and disagreement into the field of the taxonomist and has thrown

difficulty into the path of the microbiologist. Unfortunately, the field and the path are filled with such debris. In many instances original cultures have been lost and no one knows exactly what was being dealt with. Many older descriptions are wholly inadequate and so ambiguously expressed that they might fit a dozen different species. On trying to duplicate them no exact descriptions of the methods used are to be found. Many "definitions" of genera (groups of similar species) are very indefinite and are merely vague general descriptions. For example, in describing a genus, it helps little to say that *some* species in a genus are motile, *many* are gram-negative, *most* are encapsulated, *several* are pigmented, a *few* forms *tend to be* microaerophilic, and so on.

Even if a complete and generally acceptable system were finally agreed upon, a new difficulty would arise because microorganisms are variable and an organism having one set of characters today may have others tomorrow. This would be a serious obstacle by itself, but it is still further complicated by the fact that some sorts of microorganisms may have some of the characters of others. The classifists and taxonomists (those who bring order out of such seeming chaos) finally set up arbitrary divisions among the creatures they are classifying and rule that the possession or absence of certain characters shall differentiate between such and such organisms. This is convenient until it is found that certain of the creatures have lost some of those characters, but have acquired others. Finally, a man-made difficulty arises when it is found that by means of irradiation with ultraviolet light and by means of transfers of genetic material (transformations, transductions, genetic recombinations, etc.) and other means it is possible to transmute one "species" of microorganism into another! However, such variation has definite limitations under ordinary circumstances. Certain characters of most species are reasonably constant. The fact that certain organisms may be classed as species is due to this fact. As pointed out by Skerman, "recognition of them as species has come through the gradual recognition of the constancy of certain characteristics which they display . . ." "Variants have been recognized only because certain characters were selected as representative of a species." Classification is obviously possible but it depends on cooperation among microbiologists, a knowledge of variable properties, and of the principles and problems of classification, taxonomy and nomenclature.

**Classification Schemes.** Many systems of classification have been brought forward but none has remained long without revision and enlargement. One of the first schemes for bacteria was devised by Cohn in 1872 based on morphology only. In 1897 Migula devised a scheme based not only on form but on color and some physiological characters like nitrogen fixation. Orla-Jensen in 1909 made up a system based largely on physiological properties and this has served as a model for all later schemes. The suggested scheme of Stanier and Van Niel, including the establishment of a new kingdom, the *Monera*, is most stimulating. A very excellent critique of the whole problem has been drawn up by Van Niel. Modern schemes use all known properties for classification: form, arrangement, Gram stain, motility, physiology, biochemistry, etc. The relative values of various kinds of characters and the difficulties with them and the whole philosophy of classification are very readably presented by Cowan and others.



A system widely used by American bacteriologists and which still has an international standing was formerly sponsored by the Society of American Bacteriologists. It is now published in a volume generally known as "Bergey's Manual." This classification is based on the botanical code of the International Congresses of Botanists. The Bacteriological Code of Nomenclature was approved by the International Committee on Bacteriological Nomenclature in 1947.

This system, in its 6th edition (1948), divided the entire group of bacteria (Class Schizomycetes) into 5 major subdivisions or orders, which are outlined below. There will be numerous changes from this in a new edition which is in preparation. Each order is divided into families, and these into tribes, genera and species. Groups of similar species constitute genera; groups of similar genera constitute tribes, and so on. The names of orders end with the suffix *ales*; names of families with *aceae*; names of tribes end with *eae*.

Some names of species and genera are changed with successive revisions of the systems of classification, and different names are therefore sometimes used by different authors for the same organism, or two different organisms may be called by the same name. Thus there is some confusion in bacteriological literature. This is one of the signs of progress, like the confusion attendant on the repair of a downtown thoroughfare.

#### OUTLINE CLASSIFICATION OF THE SCHIZOMYCETES AND RELATED MICROORGANISMS\*

##### Class SCHIZOMYCETES Nägeli

##### ORDER I. EUBACTERIALES Buchanan

##### SUBORDER I. EUBACTERIINEAE Breed, Murray and Hitchens

##### Family I. *Nitrobacteriaceae* Buchanan

##### Tribe I. *Nitrobacterieae* Winslow et al.

- Genus I. *Nitrosomonas* Winogradsky
- Genus II. *Nitrosococcus* Winogradsky
- Genus III. *Nitrospira* Winogradsky
- Genus IV. *Nitrocystis* Winogradsky
- Genus V. *Nitrosogloea* Winogradsky
- Genus VI. *Nitrobacter* Winogradsky
- Genus VII. *Nitrocystis* Winogradsky

##### Tribe II. *Hydrogenomonadeae* Pribram

- Genus I. *Hydrogenomonas* Orla-Jensen

##### Tribe III. *Thiobacilleae* Bergey, Breed and Murray

- Genus I. *Thiobacillus* Beijerinck

##### Family II. *Pseudomonadaceae* Winslow et al.

##### Tribe I. *Pseudomonadeae* Kluyver and Van Niel

- Genus I. *Pseudomonas* Migula
- Genus II. *Xanthomonas* Dowson
- Genus III. *Methanomonas* Orla-Jensen
- Genus IV. *Acetobacter* Beijerinck
- Genus V. *Protaminobacter* den Dooren de Jong
- Genus VI. *Mycoplana* Gray and Thornton

##### Tribe II. *Spirilleae* Kluyver and Van Niel

- Genus I. *Vibrio* Müller
- Genus II. *Desulfovibrio* Kluyver and Van Niel
- Genus III. *Cellvibrio* Winogradsky

\* From Bergey's Manual of Determinative Bacteriology. 6th Ed., 1948. The Williams and Wilkins Co., Baltimore, Md.

- Genus IV. *Cellfalcicula* Winogradsky
- Genus V. *Thiospira* Vislouch
- Genus VI. *Spirillum* Ehrenberg
- Family III. *Azotobacteriaceae* Bergey, Breed and Murray
  - Genus I. *Azotobacter* Beijerinck
  - Appendix: Genus A. *Azotomonas* Stapp
- Family IV. *Rhizobiaceae* Conn
  - Genus I. *Rhizobium* Frank
  - Genus II. *Agrobacterium* Conn
  - Genus III. *Chromobacterium* Bergonzini
- Family V. *Micrococcaceae* Pribram
  - Genus I. *Micrococcus* Cohn
  - Appendix: Genus A. *Methanococcus* Kluver and Van Niel
  - Genus B. *Pediococcus* Balcke
  - Genus II. *Gaffkya* Trevisan
  - Genus III. *Sarcina* Goodsir
  - Subgenera:
    - Zymosarcina* Smit
    - Methanosarcina* Kluver and Van Niel
    - Sarcinococcus* Breed
    - Sporosarcina* Orla-Jensen
- Family VI. *Neisseriaceae* Prévot
  - Genus I. *Neisseria* Trevisan
  - Genus II. *Veillonella* Prévot
- Family VII. *Lactobacteriaceae* Orla-Jensen
  - Tribe I. *Streptococcae* Trevisan
    - Genus I. *Diplococcus* Weichselbaum
    - Genus II. *Streptococcus* Rosenbach
    - Genus III. *Leuconostoc* Van Tieghem
  - Tribe II. *Lactobacilleae* Winslow et al.
    - Genus I. *Lactobacillus* Beijerinck
    - Subgenera:
      - Thermobacterium* Orla-Jensen
      - Streptobacterium* Orla-Jensen
      - Betabacterium* Orla-Jensen
    - Appendix: Genus A. *Leptotrichia* Trevisan
    - Genus II. *Microbacterium* Orla-Jensen
    - Genus III. *Propionibacterium* Orla-Jensen
    - Genus IV. *Butyribacterium* Barker
- Family VIII. *Corynebacteriaceae* Lehmann and Neumann
  - Genus I. *Corynebacterium* Lehmann and Neumann
  - Genus II. *Listeria* Pirie
  - Genus III. *Erysipelothrix* Rosenbach
- Family IX. *Achromobacteriaceae*
  - Genus I. *Alkaligenes* Castellani and Chalmers
  - Genus II. *Achromobacter* Bergey et al.
  - Genus III. *Flavobacterium* Bergey et al.
- Family X. *Enterobacteriaceae* Rahn
  - Tribe I. *Eschericheae* Bergey, Breed and Murray
    - Genus I. *Escherichia* Castellani and Chalmers
    - Genus II. *Aerobacter* Beijerinck
    - Genus III. *Klebsiella* Trevisan
    - Appendix: Genus A. *Paracolobactrum* Borman, Stuart and Wheeler
  - Tribe II. *Erwineae* Winslow et al.
    - Genus I. *Erwinia* Winslow et al.
  - Tribe III. *Serrateae* Bergey, Breed and Murray
    - Genus I. *Serratia* Bizio emend. Breed and Breed
  - Tribe IV. *Proteae* Castellani and Chalmers
    - Genus I. *Proteus* Hauser

- Tribe V. *Salmonelleae* Bergey, Breed and Murray  
 Genus I. *Salmonella* Lignières  
 Genus II. *Shigella* Castellani and Chalmers
- Family XI. *Parvobacteriaceae* Rahn  
 Tribe I. *Pasteurelleae* Castellani and Chalmers  
 Genus I. *Pasteurella* Trevisan  
 Genus II. *Malleomyces* Hallier  
 Genus III. *Actinobacillus* Brumpt  
 Appendix: Genus A. *Donovania* Anderson et al.
- Tribe II. *Brucelleae* Bergey, Breed and Murray  
 Genus I. *Brucella* Meyer and Shaw
- Tribe III. *Bacteroidae* Breed, Murray and Hitchens  
 Genus I. *Bacteroides* Castellani and Chalmers  
 Genus II. *Fusobacterium* Knorr  
 Appendix: Genus A. *Fusiformis* Hoelling
- Tribe IV. *Hemophileae* Winslow et al.  
 Genus I. *Hemophilus* Winslow et al.  
 Genus II. *Moraxella* Lwoff  
 Genus III. *Noguchia* Olitsky, Syverton and Tyler  
 Genus IV. *Dialister* Bergey et al.  
 Appendix: Tribe *Mimeae* DeBord
- Family XII. *Bacteriaceae* Cohn  
 Genus I. *Bacterium* Ehrenberg  
 Subgenera:  
*Kurthia* Trevisan  
*Cellulomonas* Bergey et al.  
*Saccharobacterium* Sickles and Shaw  
*Agarobacterium* Angst  
*Photobacterium* Beijerinck  
*Methanobacterium* Kluyver and Van Niel  
 Appendix: Suborder *Eubacteriineae*: Overlooked Species and Synonyms
- Family XIII. *Bacillaceae* Fischer  
 Genus I. *Bacillus* Cohn  
 Genus II. *Clostridium* Prazmowski
- ✓ SUBORDER II. CAULOBACTERIINEAE Breed, Murray and Hitchens  
 Family I. *Nevskiaceae* Henrici and Johnson  
 Genus I. *Nevskia* Famintzin
- Family II. *Gallionellaceae* Henrici and Johnson  
 Genus I. *Gallionella* Ehrenberg
- Family III. *Caulobacteriaceae* Henrici and Johnson  
 Genus I. *Caulobacter* Henrici and Johnson
- Family IV. *Siderocapsaceae* Pribram  
 Genus I. *Siderocapsa* Molisch  
 Genus II. *Sideromonas* Cholodny
- Appendix: Family *Pasteuriaceae* Laurent  
 Genus I. *Pasteuria* Metchnikoff  
 Genus II. *Blastocaulis* Henrici and Johnson
- ✓ SUBORDER III. RHODOBACTERIINEAE Breed, Murray and Hitchens  
 Family I. *Thiorhodaceae* Molisch  
 Genus I. *Thiosarcina* Winogradsky  
 Genus II. *Thiopedia* Winogradsky  
 Genus III. *Thiocapsa* Winogradsky  
 Genus IV. *Thiodictyon* Winogradsky  
 Genus V. *Thiothece* Winogradsky  
 Genus VI. *Thiocystis* Winogradsky  
 Genus VII. *Lamprocystis* Schroeder  
 Genus VIII. *Amoebobacter* Winogradsky  
 Genus IX. *Thioplycoccus* Winogradsky  
 Genus X. *Thiospirillum* Winogradsky

- Genus XI. *Rhabdomonas* Cohn
- Genus XII. *Rhodotheca* Molisch
- Genus XIII. *Chromatium* Perty
- Family II. *Athiorhodaceae* Molisch
  - Genus I. *Rhodopseudomonas* Kluyver and Van Niel emend. Van Niel
  - Genus II. *Rhodospirillum* Molisch
- Family III. *Chlorobacteriaceae* Geitler and Pascher
  - Genus I. *Chlorobium* Madson
  - Genus II. *Pelodictyon* Lauterborn
  - Genus III. *Clathrochloris* Geitler
  - Genus IV. *Chlorobacterium* Lauterborn
  - Genus V. *Chlorochromatium* Lauterborn
  - Genus VI. *Cylindrogloea* Perfiliev

✓ ORDER II. ACTINOMYCETALES Buchanan

- Family I. *Mycobacteriaceae* Chester
  - Genus I. *Mycobacterium* Lehmann and Neumann
- Family II. *Actinomycetaceae* Buchanan
  - Genus I. *Nocardia* Trevisan
  - Genus II. *Actinomyces* Harz
- Family III. *Streptomycetaceae* Waksman and Henrici
  - Genus I. *Streptomyces* Waksman and Henrici
  - Genus II. *Micromonospora* Orskov

✓ ORDER III. CHLAMYDOBACTERIALES Buchanan

- Family I. *Chlamydobacteriaceae* Migula
  - Genus I. *Sphaerotilus* Kützing
  - Genus II. *Clonothrix* Roze
  - Genus III. *Leptothrix* Kützing
- Family II. *Crenothricaceae* Hansgird
  - Genus I. *Crenothrix* Cohn
- Family III. *Beggiatoaceae* Migula
  - Genus I. *Thiothrix* Winogradsky
  - Genus II. *Beggiatoa* Trevisan
  - Genus III. *Thiospirillopsis* Uphof
  - Genus IV. *Thioploca* Lauterborn
- Appendix: Family *Achromatiaceae* Massart
  - Genus I. *Achromatium* Schewiakoff
  - Genus II. *Thiovulum* Hinze
  - Genus III. *Macromonas* Utermöhl and Koppe

Appendix: Order *Caryophanales* Peshkoff

- Family I. *Pontothricaceae* Peshkoff
  - Genus I. *Pontothrix* Nadson and Krassilnikow
- Family II. *Arthromitaceae* Peshkoff
  - Genus I. *Arthromitus* Leidy
  - Genus II. *Coleomitus* Duboscq and Grassé
- Family III. *Oscillospiraceae* Peshkoff
  - Genus I. *Oscillospira* Chatton and Perard
- Family IV. *Caryophanaceae* Peshkoff
  - Genus I. *Caryophanon* Peshkoff

✓ ORDER IV. MYXOBACTERIALES Jahn

- Family I. *Cytophagaceae* Stanier
  - Genus I. *Cytophaga* Stanier
- Family II. *Archangiaceae* Jahn
  - Genus I. *Archangium* Jahn
  - Genus II. *Stelangium* Jahn
- Family III. *Sorangiaceae* Jahn
  - Genus I. *Sorangium* Jahn
- Family IV. *Polyangiaceae* Jahn
  - Genus I. *Polyangium* Jahn
  - Genus II. *Synangium* Jahn

- Genus III. *Melittangium* Jahn
- Genus IV. *Podangium* Jahn
- Genus V. *Chondromyces* Berkeley and Curtis
- Family V. *Myxococcaceae* Jahn
  - Genus I. *Myxococcus* Thaxter
  - Genus II. *Chondrococcus* Jahn
  - Genus III. *Angiococcus* Jahn
  - Genus IV. *Sporocytophaga* Stanier
- ✓ ORDER V. SPIROCHAETALES Buchanan
  - Family I. *Spirochaetaceae* Swellengrebel
    - Genus I. *Spirochaeta* Ehrenberg
    - Genus II. *Saprospira* Gross
    - Genus III. *Cristispira* Gross
  - Family II. *Treponemataceae* Schaudinn
    - Genus I. *Borrelia* Swellengrebel
    - Genus II. *Treponema* Swellengrebel
    - Genus III. *Leptospira* Noguchi

**Species and Genus.** In bacteriology the terms species and genus are used, but the concept of these is somewhat vague as there is little knowledge of genetics in relation to bacteria and no knowledge of evolutionary and phylogenetic relationships. Species of animals and plants are generally established on the basis of their inability to cross-fertilize. But cross-fertilization in bacteria is very doubtful indeed, except possibly in one strain of *Escherichia coli*, and no other sexual phenomena of recognized types occur in bacteria. How then define a species? In bacteriology a species is theoretically a single kind of bacterium, all individual cells of which are identical or nearly so. In actuality this identity of cells rarely exists. In any culture of a given species mutant cells may be found which, while having the outward form, staining properties and other characters of most of the cells in the group, possess different metabolic properties, different antigenic composition, and so on. Usually these differences are not extreme and may represent only temporary fluctuations from the principal type. It must be remembered that many ordinary test-tube cultures of a species consist of billions of individuals and represent a "population" many "generations" old even in 18 hours of growth. It would be strange if there were the high degree of uniformity among microorganisms that is seen in inorganic crystals, for example.

When two bacteria have one or more *well-marked* morphological, metabolic or other differences between them which are *constant*, the two may be regarded as distinct species. Here we run into an arbitrary difficulty. Who is to determine what character or characters shall constitute "well-marked," and of sufficient distinction to be the basis for differentiation between species or genera? The same differences may be used as a basis of generic or tribal or even familial distinction between some other kinds of bacteria. Bacterial species, therefore, are rather ill defined.

The concept of genera is, in many instances, equally nebulous. A genus is theoretically, and ideally, a group of species all of which bear sufficient resemblance to one another to be considered closely related and easily distinguishable from members of other groups or genera. The boundaries of some genera are sharply defined by as few as three characteristics, as in the genus *Bacillus*: (1) aerobic; (2) spore-forming; (3) rods. These are very *definite*, *distinct*, *constant* and *readily determined* characters. The boundaries of other

genera are sometimes more difficult of definition; for example, the genera *Salmonella*, *Escherichia*, *Shigella*, and *Aerobacter*, all of which are nonspore-forming, gram-negative, facultative, aerobic rods of identical appearance, non-pigmented and fermenting glucose. An organism of one genus may thus possess several of the important (?) characters of two or three or more other genera, and its proper allocation to one of these is often difficult and must be decided on an arbitrary basis.

**Strains.** A term frequently used in microbiology is "strain." A strain of microorganisms is a particular example, specimen or "culture" of a given species. For example, we might isolate a culture of *Micrococcus pyogenes*, var. *aureus*, from Mr. Jones and, later, another culture of the same species from Miss Smith. We may call the first culture the "Jones" strain; the latter the "Smith" strain. They may or may not show temporary or minor differences which are referred to as "strain differences."

**Clones.** A clone is a strain of microorganism derived from a single cell and, therefore, asexually propagated. Can sex appear in a clone? How?

**Bacterial Nomenclature.** The selection of the proper name for an organism requires a knowledge of the rules of nomenclature, of previously used names, and an exhaustive knowledge of the characteristics of the organism and of similar organisms already named. The name of an organism is (or should be) a descriptive symbol. It should convey a definite idea of the organism named. This saves words and time and confusion. But it requires meticulous care not to use the same name for different organisms, and to describe the organism itself fully and accurately.

In naming a bacterium, certain definite conventions are followed. Each species is allowed a "first" and "last" name only. The two-name scheme is called the binomial system and originated in 1760 under the leadership of Linnaeus. The first name of a bacterium refers to the genus, and is usually a Latin or latinized word generally a noun, based on the morphology of the organism, the name of the discoverer or some other distinguishing character, habitat, etc. It is written with a capital initial letter. The last name is the species name and is usually descriptive of the noun, referring to its color, source, disease production, discoverer or some other distinguishing point. It is not capitalized. Genus and species names are generally italicized. For example, the name *Bacillus anthracis* indicates that the organism is a spore-bearing aerobic rod (properties of the genus *Bacillus*), while *anthracis* calls attention to the fact that this species of the genus *Bacillus* produces the disease anthrax. The name *Spirillum rubrum* shows that the organism is a true, saprophytic bacterium, rigid, spiral in structure, non-sporeforming, motile, and gram-negative (all properties of the genus *Spirillum*), and that the species named is characterized by a red color (*rubrum*). The name *Clostridium novyi* indicates a gram-positive, sporeforming, rod-shaped organism, saprophytic or parasitic, and restricted to growth in the *total absence* of free oxygen. These are properties of the genus *Clostridium*. This particular species bears the name *novyi* in honor of Dr. F. G. Novy of the University of Michigan, who discovered the organism and its relation to a disease, gas gangrene. The practice of using personal names for newly discovered species of bacteria is obsolescent, although many generic names are derived from the discoverers or original students of the genus; for example the genus *Salmonella*, from an

American bacteriologist named Salmon; *Escherichia* from Escherich, a famous German bacteriologist.

In writing of bacteria, it is customary to abbreviate the generic name, using only the initial letter if it is clear what genus is meant; for example *B. anthracis* for *Bacillus anthracis*. Sometimes the abbreviation may be longer, as *Br. abortus* for *Brucella abortus*. Medical bacteriologists often dispense with these rules and designate organisms by the disease with which they are most frequently associated, for example, meningococcus, pneumococcus, typhoid bacillus, etc. This is a convenient but loose custom not in accord with the rules of nomenclature.

**Type Species.** There are certain central types of bacteria as, for example, *Streptococcus pyogenes*, *Bacillus subtilis*, *Clostridium butyricum*, and the like. Each of these is a well-known, thoroughly-studied, easily-identifiable species representative of a genus or a group of species. It is spoken of as the *type species* of that genus or group of organisms. Usually it is the first-described member of the species. To every experienced bacteriologist each of these names conveys a very definite idea as to the characters of the group. However, ill-defined, partly-studied organisms distinguishable from the type species, or from each other, only with the greatest difficulty or not at all, are often included in such groups. On the other hand, organisms differing so markedly from the type species that the relationship seems very vague may also be included. Endless arguments often arise concerning such matters.

**Biochemistry and Classification.** With the increase in knowledge of chemistry in general, there came an increase in knowledge of the biochemistry of bacteria. After Koch had showed the way to pure-culture study, it became possible to differentiate between morphologically identical organisms by their biochemical properties, such as ability to ferment certain sugars, and so on. These properties soon found their way into classification as the bases of subdivisions of previously large and heterogeneous morphological groups. Today many important distinctions are based on biochemical properties of morphologically indistinguishable organisms.

**Antigenic Structure and Classification.** Briefly, antigens are molecules, mainly of proteins but also of some carbohydrates which, when gaining entrance to the deeper tissues of the body, stimulate those tissues to produce similar molecules called *antibody*. Antibodies are found in the blood serum.

Antigen and antibody molecules are structurally reciprocally related, as mirror to image or key to lock. Antibodies combine with antigens which stimulate their production. All antigen-antibody reactions are highly *specific*; that is, by virtue of the curious key-lock relationship, the antibody produced in response to a given antigen will combine *only* with that antigen. This specificity offers a tool for differentiating microorganisms by means of antibodies evoked from tissues by antigens of the microorganisms, such as flagellar proteins, capsular polysaccharides, etc. The method, often called *antigenic analysis*, has been extensively used. In attempts to introduce still greater accuracy into systems of classification, immunology\* has been drawn upon. Striking biochemical and antigenic differences between organisms formerly believed to be identical have been found. These are often used to

\* Immunology is in part the study of antigen-antibody relationships.

formulate new and finer and more accurately-defined groupings. For example, all pneumococci were thought to be identical until it was found that the polysaccharides constituting their capsules fell into some 75 or more distinct antigenic groups which are called *types*: Type I, Type II, etc. Such types, based on antigenic differences are often spoken of as *serotypes* because the antibodies used to determine the different types are found in serum. An analogous situation was found among hemolytic streptococci. We now speak of Group A or Group B hemolytic streptococci, etc., depending on the antigenic nature of their carbohydrates.

## REFERENCES

- Bergey, et al.: Manual of Determinative Bacteriology. 6th ed. Williams & Wilkins Co., Baltimore, Md., 1948.
- Buchanan, R. E.: Taxonomy. Ann. Rev. Microbiol., 1955, 9:1.
- Gould, S. W.: Permanent numbers to supplement the binomial system of nomenclature. Am. Sci., 1954, 42:269.
- International Bul. of Bact. Nomencl. and Taxon. Iowa State College Press, Ames, Iowa.
- Knight, B. C. J. G., and Standfast, A. F. B., Editors: Symposium on the principles of microbial classification. J. Gen. Micr., 1955, 12:314.
- Skerman, V. B. D.: A mechanical key for the generic identification of bacteria. Bact. Rev., 1949, 13:175.
- Van Niel, C. B.: The classification and natural relationships of bacteria. Cold Spring Harbor Symp. on Quant. Biol., 1946, 11:285.



## Effect of Chemical and Physical Agents on Microorganisms

THE WIDE distribution of life on the globe today is due to the tendency of living things to change or *mutate* so that they may, if the properties of the mutant form are appropriate, live in new situations or under new conditions to which the older forms were not adapted. The mutants may, on the other hand, be so ill-adapted, even to their initial situation, that conditions of life prevent their multiplication or kill them. These two phenomena: (a) mutation followed by (b) selection of those able to multiply in the environment are the basis of evolution. As a result of evolutionary processes microorganisms have appeared, capable of utilizing what seem to us the most indigestible of foods (pure sulfur, kerosene, carbolic acid, etc.), and thriving in the (to us) most remote, dismal and uninhabitable places, such as the depths of ocean ooze, subterranean slimes, petroleum wells, hot sulfur springs and arctic seas, as well as inside animal intestines, and in the tissues of animals and plants. So complete and highly specialized is the adaptation that many forms of microorganisms perish when transferred suddenly to other environments. In this chapter we shall discuss some factors in the environment which affect microorganisms.

### TEMPERATURE

**Temperature and Growth.** Temperature is one of the most important factors influencing all forms of life. The relations of temperature to the growth of bacteria are somewhat complex. Many species indigenous to the soil, waters, and the animal body grow well at temperatures from 20° to 40° C (human body temperature is 37° C). Species growing well at such temperatures are called *mesophilic* (moderation-loving). Some soil and marine bacteria and species pathogenic for fish or plants grow best at temperatures very little above the freezing point (4° to 10° C) and these are referred to as *psychrophilic* (cold-loving). Certain marine forms, adapted to life at around 4° C, will die if held at about 30° C for more than a few minutes. These could be called *obligate psychrophils*, i.e., they are limited to low temperature as a condition of life. There are, in addition, several species of bacteria which thrive only at high temperatures (45° to 75° C). Such species are called *thermophilic* (heat-

loving). Some species of thermophilic bacteria are found in hot sulfur springs while others occur in milk, soil, manure, and related situations.

A distinction should be made in all cases between ability to *endure* a given temperature and ability to *grow* well under the same conditions. Many bacteria, for example, can live for months or years frozen in "dry ice," yet do not grow at all. *Thermoduric* (heat-enduring) organisms *survive* well at higher temperatures, but only *thermophilic* species *grow* well under such conditions.

It is worth noting that the *optimal* temperatures for growth of nearly all microorganisms are near the *upper*, maximal limits of their range. Most microorganisms have a wide tolerance to temperatures below optimal.

**GROWTH TEMPERATURES AND ENZYMES.** It is noteworthy that the optimal and limiting growing temperatures for microorganisms, and indeed for all living cells are, in general, the optimal and limiting temperatures of their enzymes.

Enzymes are protein complexes within the cell which are basically responsible for the chemical reactions of life. Each vital chemical reaction in the cell, and there are many in each cell, is mediated by an enzyme which is specific for that reaction.

Now, enzymes have minimal, optimal and maximal reaction temperatures. Some operate best at around 10° C. Such enzymes are found in cold-blooded animals, arctic plants, psychrophils, etc. Others function best at temperatures of the mammalian body (around 37° C); some at summer, outdoor temperatures (20° C–40° C) or at temperatures of natural hot springs or the interior of piles of actively "rotting" manure (50° C–75° C). The latter are found in thermophils.

Some curious temperature effects on synthetic and other enzymes have been noted with respect to nutrition. For example, cultivated at about 41° C, a strain of *Esch. coli* was found to require, absolutely, the vitamin *pantothenic acid*. At 37° C it can synthesize this vitamin for itself. Some organisms were found to utilize certain vitamins more efficiently at lower-than-optimal temperatures. It is thus clear that, when we note a temperature as "optimal" for a given organism, we must remember that the optimal temperature may vary under different *nutritive* conditions. At optimal temperatures most of the cell enzymes act favorably for the cell. As the temperature departs from optimal, one after another enzyme ceases to function. Unless the metabolites whose formation each enzyme mediates are supplied ready-made, the cell ceases growth and may die.

At temperatures well below the optimum, enzymes function more slowly or not at all. This is, in part, because low temperatures generally cause increases in viscosity of fluids, hardening of lipids, and slower chemical reactions. Growth is retarded or inhibited, but low temperatures are not destructive as are high temperatures.

**Thermal Resistance.** With respect to mere *survival* of heat, most microorganisms in an actively-growing (*vegetative*) state are readily killed by exposures to temperatures of around 70° C for 1 to 5 minutes. A good many are killed by 10 minutes in water at as low as 54° C; the diphtheria bacillus (*Corynebacterium diphtheriae*) for example. The conditions of commercial pasteurization (62° C for 30 minutes) kill most vegetative *pathogens* in milk, including tubercle bacilli (*Mycobacterium tuberculosis*). Numerous *sapro-*

*phytes* in milk survive pasteurization. Thermophils are quite resistant, some vegetative cells surviving 80° C to 90° C for as long as 10 minutes. Boiling kills *all* non-sporeforming bacteria in a few moments.

Bacterial spores are much more resistant, as has already been pointed out. Spores may survive boiling or higher temperatures for many hours. Killing of spores is more fully discussed in the chapter on disinfection.

**THERMAL DEATH POINT.** The temperature at which all the bacteria of a given species are killed after ten minutes' exposure is sometimes spoken of as the "thermal death point" of that species. This is an inexact expression because, at a given temperature, say 70° C, the bacteria in a given culture or situation do not all die simultaneously and suddenly just as the clock registers the expiration of ten minutes. Unless the temperature is catastrophic, as when dropping a culture into a furnace, "thermal death point" merely tells us when the *last survivor* of all has expired, the number of live cells having been decreasing continuously during the exposure period. Further, in measuring thermal death point much depends on the species and numbers of bacteria originally present, the age of the bacteria, the acidity of the suspending fluid, its osmotic pressure, its composition, etc., so that the term "thermal death point" of a certain species can be correctly used only if the exact conditions of a given experiment are known. The time required to kill in a moderately acid fluid, such as tomato or orange juice for example, would be very much shorter than in a neutral or slightly alkaline fluid such as milk or serum. However, for certain purposes, where the exact conditions are carefully controlled, information on thermal death points is of practical use, as in the commercial preservation of certain foods, milk, etc.

**THERMAL DEATH TIME.** This is the length of time required to kill all of the bacteria of a certain species in a given substance at a stated temperature. The thermal death time of various species of microorganisms is influenced by the same factors that affect thermal death point, but under known conditions knowledge of thermal death time is of practical use. Thus, in pasteurizing, milk is heated to 62° C and held there for thirty minutes. This is considerably in excess of the *thermal death time, at 62° C, in market milk*, of all the *pathogenic* microorganisms known to be commonly transmitted by milk. Many non-pathogenic bacteria survive pasteurization so that pasteurized milk is safe but not necessarily sterile.

**Rate of Death.** As noted above, death of all the bacteria of any given species in a given material does not occur simultaneously unless the lethal influence be overwhelming. On the contrary, death occurs in a definite relationship to time, the rate being determined by various factors such as numbers and kind of cells initially present, temperature, moisture, acidity, etc.

Mature (not senescent) cells are more resistant than young ones. Moisture and acidity greatly increase the vulnerability of cells to heat. Cells inside of solid material such as mucus or canned meats may escape heat longer than the same cells in distilled water or in broth, because the heat does not penetrate immediately into the center of solid masses.

Most killing processes, whether by heat or chemical disinfectant, behave like a monomolecular\* chemical reaction, progressing in accordance with

\* A monomolecular reaction is one in which only one kind of molecule is broken down or changed into one or more other molecules.

the laws governing mass action. If the numbers of organisms surviving in a disinfection test are determined at various intervals, and plotted against the intervals, regular curves are formed. If the logarithms of the numbers are similarly plotted, a straight line is formed (Fig. 12-1). Whether killing processes really are monomolecular reactions is not certain. Probably they are complex reactions whose net result simulates a monomolecular reaction. Under certain influences these theoretical straight lines may be much distorted: rapid at first, slow toward the end, and vice versa.

**Extreme Cold.** Many species of bacteria are highly resistant to extremes of cold, even when in the vegetative state. In this respect they are almost unique among living things and it may be due to this that certain species of bacteria may have survived glacial epochs in the earth's history. Many species, even parasites like the typhoid organism, will survive (not grow) for weeks frozen in ice. The fragile syphilis spirochetes and numerous viruses have been maintained frozen in "dry ice" at  $-76^{\circ}\text{C}$  ( $-169^{\circ}\text{F}$ ) for years without loss of infectivity. Some species of bacteria will grow, apparently unaffected, even after subjection to the temperatures of liquid hydrogen ( $-252^{\circ}\text{C}$  or  $-486^{\circ}\text{F}$ ). Larger cells than bacteria are often entirely disrupted by the crystallization (freezing) of their fluids but some protozoa and metazoa and sperm cells have been held at  $-70^{\circ}\text{C}$  for long periods.

#### HYDROGEN ION CONCENTRATION\*

Another factor profoundly affecting microorganisms is the acidity or alkalinity (*reaction*) of the fluid in which they are suspended. In biology this is usually expressed in terms of *hydrogen ion concentration* (pH) since it is the

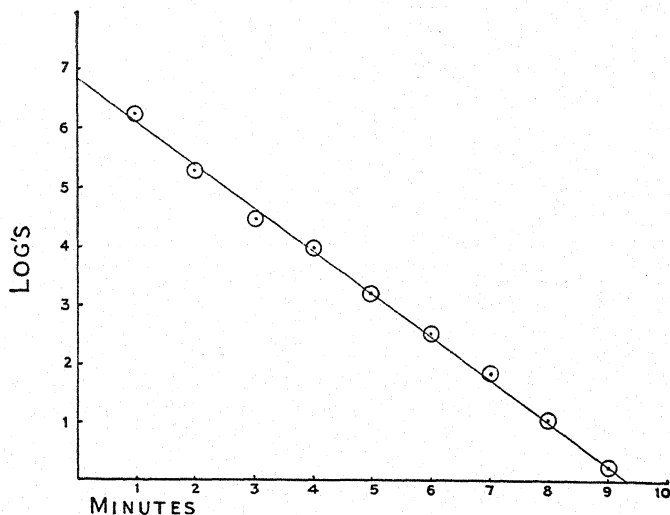


Fig. 12-1. Relation between numbers of organisms and time in a 1 per cent solution of phenol containing typhoid bacilli. The logarithms are of numbers of bacilli per ml. The straight-line curve is characteristic of the relation between time and survivors under any definitely adverse conditions when no growth occurs.

\* It is assumed the student has a working knowledge of pH and its determination. It may be reviewed in any good textbook of elementary physics.

concentration of dissociated or *ionized* hydrogen (or hydroxyl) that determines the *biologically-effective* acidity (or alkalinity) of a given solution.

#### pH IN MICROBIOLOGY

It is important for the microbiologist to remember that, as a rule, increases in temperature cause increases in dissociation of acid hydrogen so that a solution which is neutral or slightly alkaline at room temperature (about 22° C) may become definitely acid at incubator temperature (37° C). If a solution is prepared at a definite pH while near the boiling point, it will be more alkaline when cool. It is also important to note that a change in pH equivalent to 1 on the pH scale, represents a 10-fold change in hydrogen ion concentration, while a change from pH 7.0 to 7.3 represents a 2-fold change (Table 8).

**Table 8.** *Relationships of Hydrogen Ion Concentrations Expressed in Various Ways.*

REACTION	FRACTION OF NORMALITY*	HYDROGEN IONS PER LITER (grams)	LOGARITHMS OF H ION CONCENTRATIONS	EXPRESSED AS pH
Acid.....	N/1	1.0	— 0	0.0
Acid.....	N/10	0.1	— 1	1.0
Acid.....	N/100	0.01	— 2	2.0
Acid.....	N/1,000	0.001	— 3	3.0
Acid.....	N/10,000	0.000,1	— 4	4.0
Acid.....	N/100,000	0.000,01	— 5	5.0
Acid.....	N/1,000,000	0.000,001	— 6	6.0
Neutral.....	Pure water	0.000,000,1	— 7	7.0
Alkaline.....	N/1,000,000	0.000,000,01	— 8	8.0
Alkaline.....	N/100,000	0.000,000,001	— 9	9.0
Alkaline.....	N/10,000	0.000,000,000,1	— 10	10.0
Alkaline.....	N/1,000	0.000,000,000,01	— 11	11.0
Alkaline.....	N/100	0.000,000,000,001	— 12	12.0
Alkaline.....	N/10	0.000,000,000,000,1	— 13	13.0
Alkaline.....	N/1	0.000,000,000,000,01	— 14	14.0

\* With respect to hydrogen or hydroxyl ions.

Unfavorable influences of many sorts may be much enhanced in fluids having an acid pH. For example, coagulation by heat occurs more readily in acid solutions. Thus, milk which is only very slightly sour may curdle on being warmed.

Enzymes are as sensitive to alterations in pH as they are to temperature, or even more so. They have definite minimal, optimal and maximal zones and limits in respect to pH, just as they do to temperature. What is true of enzymes is largely true of living cells. The reaction of bacteriological culture media must, therefore, be very carefully adjusted with respect to pH, the degree of acidity depending on the bacteria to be cultivated.

In addition to controlling enzyme action the pH undoubtedly affects the state of colloidal suspension of protoplasm, the permeability of cell wall and

membrane, and indeed, every important physiological function. The vast majority of living forms, plant, animal, and microorganismal, have an optimal pH near the neutral point of pH 7. As with temperature, some species have become adapted to grow at a pH considerably at variance with this. Some of these will be mentioned later on. A range from about pH 6.0 (slightly acid) to pH 8.0 (slightly alkaline) includes the optimal ranges of the vast majority of species.

#### BUFFERS AND BUFFER ACTION

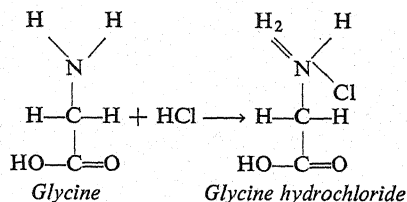
In the ordinary titration of, let us say, 500 ml of N/10 acid with N/10 alkali in distilled water, using phenol red as indicator, there comes a point in the titration when a single drop of N/10 alkali from the buret changes the whole volume of fluid from distinctly yellow or acid (pH around 3.0) to definitely alkaline as evidenced by the appearance of a deep magenta color (pH around 10). Thus, a single drop of N/10 alkali (or acid) produces a very large alteration in pH.

Now, it is often necessary in microbiology to change the pH of culture fluids ("adjust the pH"). In attempting an adjustment of the pH of bacteriological media such as meat-infusion broth it is found that, unlike the titration of the aqueous solutions of acid or alkali, no sharp end point is reached. The change from an acid to an alkaline reaction or vice versa is very gradual, requiring the continuous addition of relatively large amounts of acid or alkali; commonly thousands of times as much N/10 acid or alkali as an aqueous solution of inorganic acid or base. In other words, even at, or near, the neutral point, the solution being titrated shows a marked tendency to resist any change in its reaction. This is due to what is known as "buffer action" on the part of certain constituents of the broth.

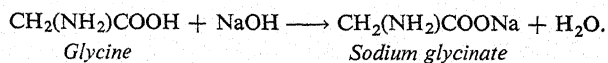
These buffer substances, neither strongly acid nor alkaline in themselves, combine with the acid or alkali as fast as it is added and so prevent change of reaction of the medium until they are all used up. The same sort of buffer actions maintain the pH inside every living cell within very narrow limits and is absolutely essential to life.

There are several sorts of substances which have the property of acting as buffers. Important among these, from a biological standpoint, are amino acids and their compounds, the polypeptides, proteoses, proteins, etc. These are neither strongly acid nor alkaline but will combine with either acid or alkali.

To cite a simple example of buffer action by an amino acid, we may note that glycine combines with HCl or NaOH as follows:

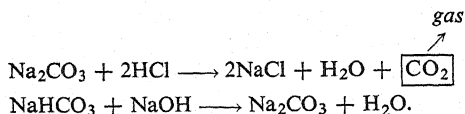


or,



One of the most commonly used buffers in biological laboratories is a mixture of the monobasic and dibasic phosphates,  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ , in aqueous solution. These dissociate relatively little in aqueous solution. When acid is added the dibasic salt absorbs  $\text{H}^+$ , changing into the monobasic and forming a potassium salt with the acid. When alkali is added, the monobasic salt gives up  $\text{H}^+$  to form  $\text{H}_2\text{O}$  with the  $\text{OH}^-$ , and the incomplete salt combines with the cation of the alkali.

Other important buffer constituents of organic media are carbonates and bicarbonates:



Lumps of marble ( $\text{CaCO}_3$ ) are sometimes added to cultures containing fermentable substances like glucose, sucrose or lactose, to maintain the pH unchanged, that is, to neutralize the acid produced by fermentation. Otherwise the organisms in the culture would quickly cease growth or die. Colloidal substances such as charcoal, precipitates of phosphates, kaolin, etc., which adsorb hydrogen or hydroxyl ions directly, are also active buffers and are used in biological systems.

#### OTHER IONS

The hydrogen and hydroxyl ions are not by any means the only ones having a marked influence on microorganisms. The well-being and life of all cells is dependent on the absence or presence, and suitable concentration, of ions of many different sorts.

**Cations.** Ions of heavy metals, such as gold, silver, lead, nickel, tellurium, copper, mercury, and iron are usually more toxic to bacteria than are ions of light metals, such as sodium, potassium, and calcium. However, generalizations are difficult. Toxicity of cations depends greatly on the species of microorganism being dealt with; the presence of chelating\* agents and other substances (such as  $\text{H}_2\text{S}$ ) with which the metal ions can combine; pH; etc. As a rule, chlorides of light metals like sodium, potassium, lithium, strontium, magnesium, calcium and of ammonia are harmless and even desirable and stimulating to most bacteria in concentrations up to about 1.5 per cent. On the other hand, chlorides of many heavy metals (Hg, Cu, Fe, Pb, Zn) usually exert definitely harmful effects, even in very low concentrations ( $\text{HgCl}_2$ , 0.0007%).

The less toxic salts act favorably in a great variety of ways, sometimes by effectively suppressing the ionization of unfavorable substances, or by reacting with these substances so as to prevent them from affecting bacteria unfavorably. For example, the presence of calcium chloride is effective in reducing the toxicity of sodium oxalate by forming an insoluble precipitate of calcium oxalate, thus removing the toxic oxalate radicle from the solution.

\* From the Greek word, *chela*, for claw. Chelating agents are generally thought of as organic compounds which form a complex compound with a heavy metal, enclosing it in a molecular, ring-like structure as though held in a claw. The position of iron in the porphyrin group of hemin is a typical example (Fig. 12-2).

The magnesium ion ( $Mg^{++}$ ) appears to be of special importance. It has been shown to lower the toxicity of Ni, Co, Zn and Mn for several species of bacteria. It appears to be an essential factor for some species, having a controlling effect on cell fission. It has already been mentioned as having a critical relationship to the Gram stain. Many ions, including  $Mg^{++}$ , probably act also by affecting the permeability of the cell membrane and its electrical charge.  $Mg^{++}$  is known also to be necessary to the functioning of several important enzymes in many bacterial species.

It appears that certain divalent cations ( $Ca^{++}$ ,  $Mg^{++}$ ,  $Sr^{++}$ ) are essential to the penetration of bacteriophage into staphylococci (*Micrococcus*), possibly by activating an enzyme in the tail of the phage particle. Cobalt ( $Co^{++}$ ), also, is apparently essential to the action of certain enzymes in bacteria. Iron ( $Fe^{++}$ ) is involved in the activation of other enzymes. Some enzymes, on the other hand, are inhibited by heavy metal ions ( $Hg^{++}$ ,  $Cu^{++}$ ,  $Fe^{++}$  and  $Zn^{++}$ ).

**ION ANTAGONISMS.** Some cations are biologically antagonistic to each other. For example,  $Li^{++}$  and  $Zn^{++}$  are definitely toxic to certain bacteria of importance in the dairy industry (*Lactobacillus* and *Leuconostoc*). These ions appear to drive  $H^{+}$  from its normal position, and to replace it in certain enzymes. They are said to *antagonize* the  $H^{+}$ . By increasing the concentration of  $H^{+}$ , this toxicity of  $Li^{++}$  and  $Zn^{++}$  is reduced.

As will be seen later, knowledge of this sort of antagonism and competition

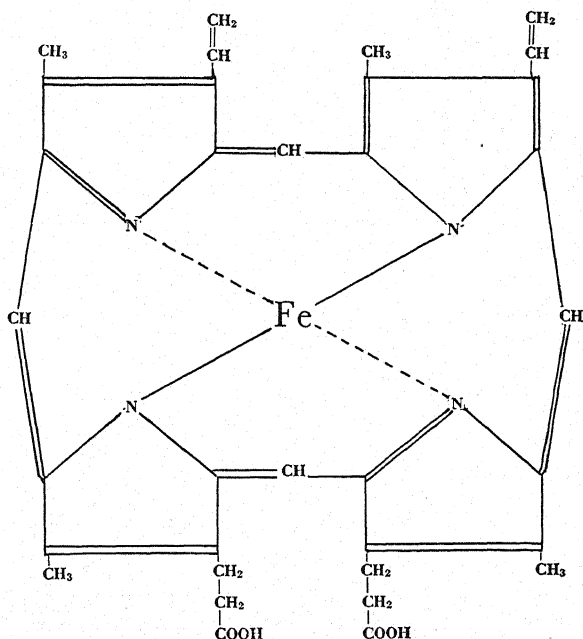


Fig. 12-2. The heme molecule.

Chelating agents, by combining with harmful metallic ions, perform a function analogous to that served by buffers in maintaining pH. Indeed, hydrogen ions are analogous to metallic ions in such situations. Some pH buffers also act as chelating agents, for example, amino acids, certain vitamins, etc.



of certain substances for key physiologic positions in living cells is of fundamental importance in disinfection, the action of chemotherapeutic drugs like sulfanilamide and antibiotics, as well as for industry and other activities. It will be well to keep it in mind.

**Anions.** The desirability or toxicity of any compound, from the standpoint of microorganisms, is determined in part by the anion as well as by the cation. In order of favorable action with regard to bacteria we may list several anions (with sodium as the cation) as follows: sulfate, tartrate, chloride, nitrate, acetate, citrate, oxalate, iodide, benzoate, salicylate, tellurite; sulfates the least toxic, tellurites the most unfavorable.

The toxicity of alkalis varies, but depends mainly on the concentration of hydroxyl ions (pOH). The cation of alkaline substances also plays its role, as it does in the action of acids and salts. This is illustrated by the fact that barium hydroxide, a much weaker (less dissociated) alkali than sodium hydroxide, is nevertheless much more toxic at the same concentration of hydroxyl ions. This is partly because the barium ion is more toxic than the sodium ion.

It is very important to remember that species vary greatly in respect to sensitivity toward various ions.

**Complete Molecules.** The biological activity of most compounds, inorganic as well as organic, depends not only on anion and cation but also on the undissociated molecule. For example, the toxic effects of benzoic and acetic acid are much greater than would be expected from equivalent concentrations of  $H^+$ . Thus, hydrochloric acid and sulfuric acid, although "strong" acids, are much less poisonous to most bacteria at a given pH than benzoic acid or acetic acid at the same pH. About 7.5 to 7.7 parts per million of the first two are required to produce the same toxic effect as 0.1 and 1.2 parts per million, respectively, of the last two. The reasons are not clear. The important point here is that one can never, with certainty, predict the effect of a substance on a given species of microorganism without some previous knowledge of biological action of that substance, supplemented by *direct experimental test*.

### MAGNETISM

The subjection of microorganisms to magnetism has not been demonstrated to exert any important influence on them.

### ELECTRICITY

The passage of an electrical current through a suspension of microorganisms probably has little effect by itself. If a current of great intensity is passed for a long time, however, electrolysis of some of the constituents of the medium will result, their nature and concentration depending on the voltage, and the composition of the medium and of the electrodes. Some of these products of electrolysis have deleterious effects. Heat, also, will be generated and, if sufficient, may kill the organisms.

**Electrophoresis.** The student familiar with colloids knows that any very minute particles, including microorganisms, when suspended in aqueous solutions, acquire an electrical charge on their surfaces. Therefore, when an electrical current is passed through a suspension of such particles, those with

a negative charge travel toward the positive electrode (anode), while those with a positive charge travel toward the negative electrode (cathode). Those with a strong charge travel faster than those with a negative charge. This migration is termed *electrophoresis*.

The electrophoretic migration of microorganisms may be observed directly with a microscope focussed on a thin, hollow cell with electrodes at each end (Fig. 12-3) or by testing for their presence in the legs of a U-tube with electrodes at each leg, the particles being introduced at the middle of the U. Other methods (paper electrophoresis, the Tiselius moving boundary method) are also used.

This phenomenon has been used in the study of the electrical charge of various microorganisms. For example, it has been shown that a modified form of yellow fever virus with special affinity for nervous tissues has electrophoretic properties which differ markedly from those of the mosquito-borne form of the virus. Pathogenic micrococci migrate differently from micrococci of little pathogenicity. Different mutants in a culture may be differentiated by their rate and direction of electrophoretic travel.

#### MOISTURE AND DESICCATION

It is obvious that abundant moisture is necessary for *active, vegetative* existence of all things. Most large plants and animals require abundant water at all times. Some plants in desert areas and many species of microorganisms, however, can survive complete drying or desiccation for long periods, though they do not grow under such conditions. Many substances such as hay, fruits, fish, meat, etc., "preserved" by drying, contain large numbers of living bacteria which are dormant but which soon grow and cause spoilage if the dried product becomes moist. On the other hand, some microorganisms, especially delicate pathogenic species, are quickly killed by drying. Spores, conidia,

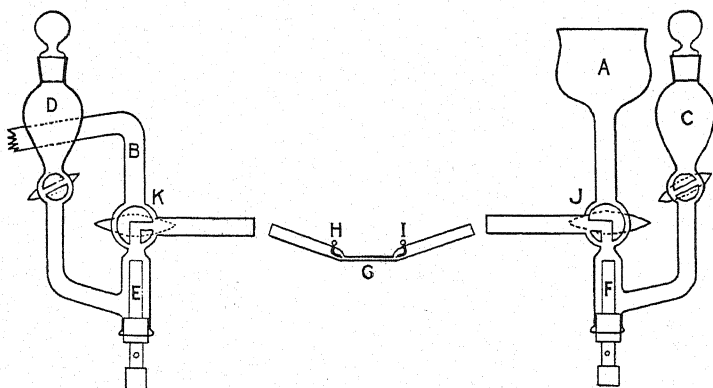


Fig. 12-3. The essential parts of an electrophoresis cell. *A*, reservoir of the suspension of bacteria to be observed. *B*, outlet tube, for passing off water used in flushing out the apparatus, etc. *C*, *D*, reservoirs for saline solution bathing the electrodes and carrying the electric current from them to the suspension of bacteria which extends from *A* through *J*, *G* and *K*. *E*, *F*, electrodes. *G*, the electrophoresis chamber proper within which actual microscopic observation of electrophoretic motion is made. *H*, *I*, glass-insulated platinum electrodes for measurement of the potential gradient across the electrophoresis cell. *J*, *K*, three-way stop cocks controlling flow of electrode fluid, bacterial suspension, or wash water.

arthrospores, etc., of course, are cells specially adapted to withstand drying for long periods without any special assistance.

**Desiccation and Vacuum.** Not only do many kinds of bacteria in a vegetative state withstand desiccation, but they may be frozen at sub-arctic temperatures and subjected to the highest possible vacuums without harm. These facts are of the greatest interest from both a philosophical and practical standpoint.

In a state of virtually complete desiccation and in a high vacuum, there must be an unimaginably small vital activity. The metabolic processes must stop almost completely since these depend largely on osmosis, diffusion, ionization and the colloidal state, all of which are dependent, in turn, on hydration. How, then, can bacteria exist frozen and desiccated in a vacuum? How can living things survive, as it seems, in the entire absence of vital activity? There are no known answers to these questions. The condition of bacteria frozen and desiccated in a vacuum, must be as near an approach to suspended animation as can be imagined.

If time (for human beings) be measured by successive events such as heart beats, thought train, action of enzymes, wars, etc., then, when these activities or their effects cease to be *observed*, time ceases *for the individual* involved. It begins again only when the individual consciousness again begins to pace exterior events or when vital activity produces the effects of age. A person deeply preoccupied by study,\* pain, play or worry, or who has been wholly unconscious, or in an abnormal mental state (delirium), in which consciousness of the course of events is temporarily lost, has a different idea of time from that of normal persons, or at least a very distorted one. An analogous situation exists for bacteria in a preservation vial; since they have no consciousness of the passage of time and appear to undergo no physiological aging, when the air and moisture are withdrawn from their environment time may be imagined as ceasing to exist for them. Time (for them) begins again only when air and fluid are added. In the vacuum jar their chronological age may become great, as measured by human standards, but they appear to remain physiologically young and unchanged even for many years during their sojourn in vacuity.

### PRESERVATION OF BACTERIA

1. **Desiccation in Vacuo.** From a practical standpoint, the survival of bacteria when desiccated in a vacuum is of great importance. In the laboratory, in the past, in order to preserve pure cultures of non-sporeforming bacteria, it was necessary to transfer the organisms frequently to fresh culture medium, incubating and then maintaining them in a vegetative state in the refrigerator. This procedure involves danger of contamination of the cultures, and alterations in character due to changing environment and mutation. These difficulties as well as loss of time, labor and cost of such methods can be eliminated where it is known that the species will withstand desiccation in vacuo. Simple methods of drying and preserving bacteria in vacuo have been described. Once preserved by desiccation in vacuo a large number of cultures may be stored in a pint fruit jar and many bacteria will survive for years. Species of pathogenic streptococci, for example, have survived unharmed for twenty-five years, while diphtheria bacilli survive for fifteen years; tubercle bacilli for 17 years. Many can survive much longer under these conditions. However, while many species of bacteria resist vacuum and drying this is not true of all.

2. **Freezing.** Many bacteria will survive in the frozen state at  $-76^{\circ}\text{C}$  that do not withstand desiccation. "Quick freezing" is done by placing the organisms in a suitable suspending fluid (blood, serum, milk, glycerol, etc.) in a small tube and immersing in a bath of "dry ice" (solid  $\text{CO}_2$ ) in alcohol. Freez-

\* For example, serious students of microbiology!

ing must be almost instantaneous to avoid the mechanical disruption which would occur if time were allowed for the formation of large ice crystals. The tube is then promptly removed to a storage chest of dry ice ( $-76^{\circ}\text{C}$ ).

This method is in routine use for the preservation of bull sperm for cattle breeders, human sperm for artificial insemination, cultures of cancer cells for cancer and virus research, animal parasites of various sorts, viruses, bacteria and so on.

**3. Freeze-drying.** A method of preserving very unstable substances such as living microorganisms, enzymes extracted from the inside of living cells, bacterial toxins, etc., all of which are inactivated on storage in a moist state or by exposure to the air, depends on (a) extreme cold; (b) rapid evaporation; and (c) vacuum. The materials to be preserved are suspended in a suitable fluid and distributed in small glass ampules connected to a high-vacuum pump. The contents of the ampules are frozen almost instantaneously by immersion in a bath of dry ice in methylcellosolve ( $-76^{\circ}\text{C}$ ). The frozen contents of the ampules are then dried from the frozen state by the application of a high-vacuum. When all vapor has been removed, the necks of the ampules are sealed with a needle of flame before destroying the vacuum. The product is a highly *lyophilic* (water-loving) powder (Fig. 12-4).

**Effects of Autolysis.** In the preservation of microorganisms by freezing, desiccation or other such means, the preserving agent must act quickly in order

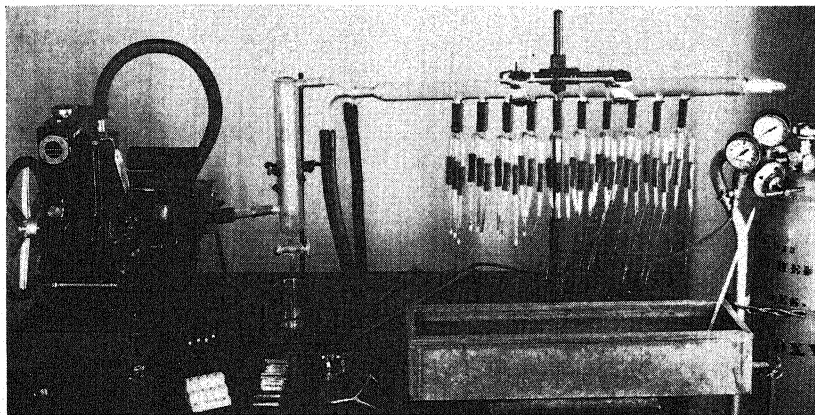


Fig. 12-4. One form of freeze-drying apparatus. The desired microorganisms from appropriate cultures are emulsified in blood serum. Each suspension is then placed inside a sterile, narrow, labeled, glass tube (mounted in wooden holder at left). The cotton plugs are re-inserted and the tubes, lubricated with a tiny drop of oil, are inserted in the rubber sleeves of the apparatus as shown. They are then immersed in the rectangular bath (below the tubes) containing alcohol or Methylcellosolve and "dry ice" ( $-76^{\circ}\text{C}$ ) where the serum freezes almost instantly. The vacuum pump is started immediately. The water is sublimated from the serum and, as the ice bath is warmed to  $-5^{\circ}\text{C}$ , drying is completed in a few minutes. Moisture is condensed and held frozen in a dry-ice trap before it reaches the vacuum pump. The lower portion of each tube is then sealed off by means of the two-jet, cross-fire, oxy-acetylene torch while evacuated. Tubes at the right of the apparatus have already been sealed off and the lower portions, with the dried suspensions, are shown laid in a row on the table. (Photo courtesy of Drs. W. C. Haynes, L. J. Wickerham and C. W. Hesseltine, Northern Utilization Research Branch, U. S. Department of Agriculture, Peoria, Ill., from Appl. Micr., 1955, vol. 3.)

to prevent deleterious effects due to autolysis. Autolysis is a process of dissolution, due to inherent enzyme-like mechanisms, which takes place in dead (or inert, live) cells. Autolysis may occur if cells undergoing preservation are held at the threshold between active and inactive existence too long.

### OSMOTIC PRESSURE

If living cells are immersed in fluids having extremely high or low osmotic pressures, water will be drawn out till the cell collapses, or forced in till the cell ruptures.

These effects of unfavorable osmotic pressures are *plasmolysis* and *plasmolysis*. Largely due to their minute volume and thin cell wall, most bacteria are not highly sensitive to variations in salt concentrations between about 0.5 and 3 per cent. Concentrations much above this may adversely affect some of the more sensitive strains. Much depends on the previous environment of the organisms. Some marine bacteria, adapted to the salinity of ocean water (about 3.5 per cent) are quite sensitive to lower or higher salinities and will not grow if the salinity is less than about 2 per cent or over about 15 per cent. On the other hand, no colonies will be formed by certain fresh-water species in media with salinities in excess of 1 per cent. Bacteria from either source, however, may be adapted to the environment of the other. Spores do not appear to be much affected by changes in osmotic pressure.

**Halophilic Organisms.** There are bacteria which have become adapted to the high salinity (around 29 per cent) of various salt waters such as the Dead Sea and the Great Salt Lake of Utah. These organisms will not grow in lake water diluted to a salinity of less than 13 per cent. Such organisms are spoken of as *halophilic* (salt-loving). On the other hand, the growth of soil, sewage or mouth bacteria is inhibited by Salt Lake water and ordinary marine bacteria are killed by a few minutes' exposure to it. There are halophilic bacteria which grow in commercial pickling brines. Some are a cause of spoilage of various commodities preserved with salt, such as fish, meat, and hides. Concentrations of salt of around 25 and 30 per cent are used in these brines. Just how much of the effects of such brines are due to ions per se and how much to osmotic pressure it is difficult to be sure. Undoubtedly osmotic effects play the major role in brines of NaCl.

Sugar syrups must be much more concentrated (around 60 per cent by weight of sugar) to achieve the same preserving effects as salt brines (around 20 per cent). This is probably due to the larger molecular size of sugar and the combined ionic effects of salt.

**Effects of Evaporation.** When a solution containing salt or non-volatile compounds is exposed to the air so that evaporation occurs *slowly*, the water, departing, leaves behind a more and more concentrated solution, the osmotic pressure becoming greater and greater. Microorganisms suspended in such a fluid are unfavorably influenced by the prolonged effect of increased osmotic tension. In the cultivation of any microorganisms, therefore, excessive evaporation of moisture from the medium is to be avoided. This may be accomplished by maintaining a very humid atmosphere in the incubator.

**Alterations in Permeability.** Even though the osmotic pressure of a given culture medium may be appropriate, there often appear in it, as a result of aging, substances that alter the permeability of the cell wall in some way so

that the cells become swollen and distorted. Such alterative substances may be the waste products (acids, alcohol, etc.) of bacterial cells which have grown in the culture. The cell wall may also become altered through aging so that it fails to function properly, very likely permitting the passage outward of important cell constituents and the passage inward of water or of deleterious compounds and probably, through localized weakenings, causing the swellings, knoblike protrusions and other irregularities often observed in bacteria in old cultures.

**Involution Forms.** Some distorted forms of bacteria are generally spoken of as "involution forms." In certain species (e.g., *V. comma*) such distortions may be brought about to a very striking extent by cultivation of the organisms on media containing high concentrations of sodium chloride.

Some modern students, however, attach importance to some of the irregular forms of bacteria as representing stages in life cycles and complicated reproductive processes. In some species they probably represent stages ("large bodies") in the life cycle of pleuropneumonia-like organisms (L forms).

### RADIANT ENERGY

**The Electromagnetic Spectrum.** As seen in Figure 12-5, electromagnetic radiations may be arranged, according to wave length, in a spectrum which includes the visible solar spectrum (red to violet), the latter being a small part of the whole system of electromagnetic waves. The physiological effects of these various radiations differ greatly, those in the shorter ranges being of most known importance. Very long waves are used in radio.

The longest waves having known physiological effect are (1) the infra-red or "heating waves." (2) The next shorter are the visible red rays, which affect the retina of the eye and also bacteriochlorophyll. (3) Then come, successively, yellow, blue and violet, which affect the retina and have photodynamic and possibly other biophysical actions; then (4) the invisible ultraviolet rays, which are shorter than the visible violet and have marked physiological and biochemical activities to be discussed. Still shorter waves of known properties are (5) x-rays ("soft" and "hard"); (6) alpha, beta and gamma rays, and finally (7) the little understood cosmic waves.

### BIOLOGICAL EFFECTS OF IRRADIATIONS

**Effects of Irradiations.** Radiant energy, especially soft x-rays and ultraviolet light which are absorbed by protoplasm, produce two principal effects on living cells: (a) lethal; (b) mutagenic. These two are not the same and often vary independently.

In irradiating a cell we may divide the events into three periods: (1) the actual instant or period of irradiation during which the ionizations and/or excitations occur; (2) the middle or latent period, after cessation of the irradiation, before the results of that irradiation become apparent. It is characteristic of biological radiation effects in general that there is a delay between irradiation and appearance of the effect: death or mutation. (3) the final period, during which the effects of the irradiation become evident.

Actively multiplying cells are most susceptible to irradiation. They are usually the most susceptible cells to other deleterious agents also: phage, antibiotics, various bacteriostatic agents, heat, etc. Bacteria in a dormant

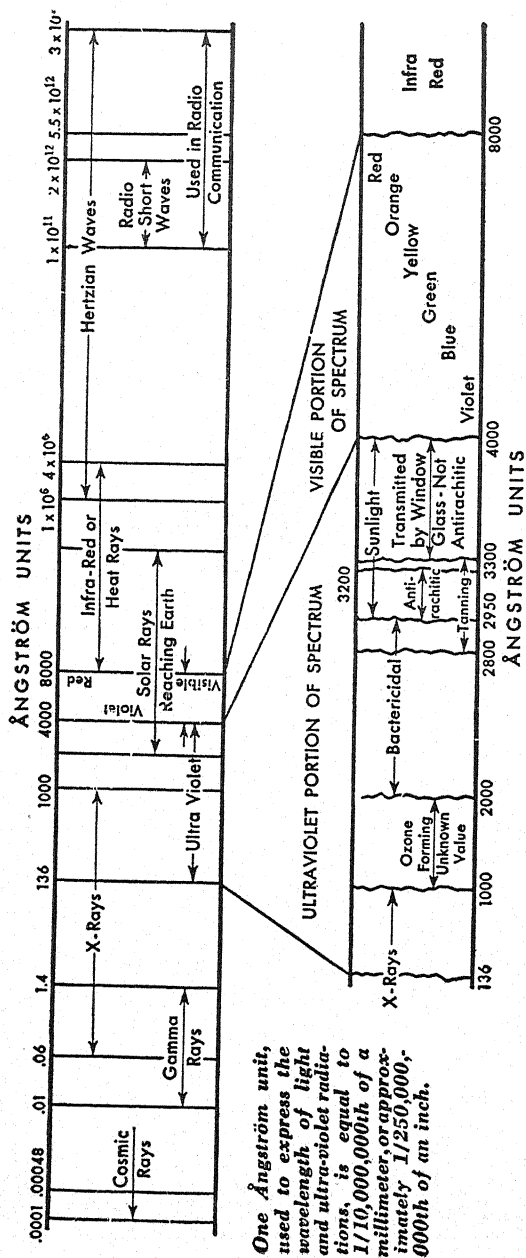


Fig. 12-5. Spectrum charts. (From The Westinghouse Sterilamp and the Rentschler-James Process of Sterilization, courtesy of the Westinghouse Electric & Manufacturing Company, Inc.)

condition, or in a mature, slow-growing stage are markedly resistant compared with fast-growing cells.

**Mechanisms of Irradiation Effects.** When radiations like soft x-rays are absorbed by living cells the energy is dissipated by removal of electrons from atoms, thus *ionizing* those atoms. These ions appear to be the agents which actually mediate the more important effects of ionizing radiations, which are: (a) genetic; and (b) lethal. Ionization may occur in a molecule of actual protoplasm, in which cases the effect (genetic or lethal) of irradiation is said to be *direct*. The direct effects appear to be due mainly to impingement of energy on *nucleoproteins*. These absorb radiant energy directly. If the ionization occurs in the materials such as enzymes, etc., dissolved in the fluids *surrounding* the actual protoplasm, the resultant effects are said to be *indirect* (genetic or lethal). Genetic effects (mutations) appear to be mainly direct effects. Lethal effects could be direct or indirect.

The effects of irradiation are complex but appear to be related largely to biological oxidations. Conversely, chemicals and physical agents which interfere with biological oxidations markedly interfere with the effects of radiation.

**ULTRAVIOLET RADIATION AND EXCITATION.** Though we speak of irradiation in a general sense it should be noted that there are differences in action and effect of the various types of radiation. These need not be detailed at present. For example, x-rays contain higher energy than ultraviolet and their effects are due to atomic disruption and consequent *ionization*. Ultraviolet rays cause *excitation* but not *ionization* and their effects are due more to a generalized distribution of *excited atoms* than to ions.

**Recovery from Irradiation.** Studies on prevention and recovery from irradiation effects are encouraging with respect to atomic warfare, accidental irradiation, sunburn, etc. Death of cells due to irradiation is often (as Mark Twain remarked about premature reports of his own death) "grossly exaggerated." Cells may be revived or, more correctly, *reactivated* after apparent death due to irradiation.

One method is exposure to visible violet light in the wave length zone around 4500 Å. Similar reactivation has been demonstrated for some viruses. This is often called *photoreactivation*. Another method is incubation of the irradiated cells in a favorable growth medium at temperatures 5° to 30° C lower than optimal. A third is by treatment of the cells with various chemical agents: indoacetate, pyruvate, and a long list of others. Many theoretical explanations of these curious phenomena exist but none is proven and we may at present only contemplate the facts and refrain from theorizing unless, like Sherlock Holmes, we expect to put the theories to experimental proof.

There are so many variables which affect the reactivation: species of microorganisms; the culture medium; age and physiological state of the cells; chemical composition of the test medium after irradiation; pH and temperature during and after irradiation, and so on.

**Special Uses of Ultraviolet Light.** Ultraviolet light, like visible light, consists of a spectrum of different wave lengths from about 4000 Ångström units (Å) down to about 200 Å (see Fig. 12-5). Those between 2800 Å and 2300 Å are most effective biologically, the most effective length depending to some extent on the species of organism tested and the conditions of test. Ultraviolet is an effective component of sunlight and produces "sun tan" and "sunburn."



It also acts on chlorophyll. Ultraviolet light has very slight powers of penetration. It passes through ordinary glass very little. It is therefore effective mainly at surfaces.

From the microbiologist's viewpoint the most obvious, but not the most interesting, biological effect is death. Microorganisms of most sorts, if irradiated with sufficiently intense ultraviolet (in the range of 2650–2750 Å) (or sunlight) for longer than a certain time (the limits depending on the organism and the environment) die. This is valuable information and has been put to practical use in medical, public health, agricultural, commercial and research applications. Radiating tubes giving off light of wave length 2537 Å are used in preventing surface spoilage of meat, tobacco, bakers' goods and the like, due to molds and bacteria. Other uses of the radiations are the disinfection of glassware in restaurants, of "conditioned" air, the air in schools, hospitals and operating rooms, and in the control of insects (see section on disinfection). Use is sometimes made of ultraviolet light in the treatment of water in swimming pools and for drinking but is not generally approved for public water supplies. A method of sterilizing serum and vaccines for use in prevention and cure of disease exposes the serum or vaccine in very thin films, to the ultraviolet light.

It is of interest to note that the effects of ultraviolet radiation are different from those of heat, since very thermostable spores are easily killed with ultraviolet light. Ultraviolet light is as effective in the absence of oxygen as in its presence.

**Ionizing Radiations and Food Preservation.** One of the most important developments in the study of lethal effects of ionizing radiations is their value in food preservation. Foods like meat pies, complete dinners, hamburgers, etc., now sold frozen (either cooked or raw) or cooked and packed in cans, may be completely sterilized and held indefinitely at room temperature, with no refrigeration, in a "fresh" state if properly irradiated *after packaging*. The prospect should give the refrigerator and "deep freeze" industries the "cold shakes" (see Chapter 44).

**Genetic Effects of Irradiations.** Among the most interesting and important effects of exposure to radiant energy, especially to ultraviolet light (aside from death) are those involving changes in genetic structures so that mutations (new species) are formed. The nature of the genetic changes induced, and the mechanisms involved, are discussed more fully in Chapter 15.

**Photodynamic Sensitization.** Visible light rays (4,000 to 8,000 Å) do not appear to exert rapidly lethal or other striking influences per se, but under certain circumstances they produce death. For example, bacteria will often grow in contact with low concentrations of bacteriostatic dyes such as eosin, fuchsin, etc., provided no light reaches them. The combined effect of light and dye for only a few minutes usually kills the organisms. Methylene blue, in the light of an ordinary electric lamp, rapidly kills certain gram-positive bacteria which withstand much higher concentrations of the dye in the dark. Gram-negative bacteria are not so affected. Other organisms, as viruses, and enzyme-like substances, proteins, etc., are also affected. The exact mechanism is not clear. It has been shown that oxygen is essential to photodynamic action, since the effects are not observed if the microorganisms are placed in a vacuum or in atmospheres devoid of oxygen.

**Sunlight.** The disinfectant ("purifying") action of sunlight has been known for centuries and has been thought to be due mainly to the ultraviolet rays in solar light. The phenomenon of photodynamic sensitization suggests that the visible light waves may play some role in the action of sunlight. It will be seen in other sections of this book that even in the visible range different colored lights have different effects. Red light, for example, is most effective in activating bacterio-chlorophyll.

The heating and drying effects of sunlight are also important in its bactericidal effect.

### HYDROSTATIC PRESSURES

If living microorganisms suspended in aqueous fluid are placed in a strong steel cylinder and a piston is pressed down upon the suspension with great force, the organisms may or may not be affected, depending on the species of organism. While some will withstand pressures of 5000 pounds per square inch without obvious injury such pressures are, in general, injurious. In the range above 9,000 pounds, death of many species occurs within one hour.

Pressures around 100,000 pounds denature proteins and inactivate enzymes. Other changes include increases in rate of some chemical reactions, diminution in volume of organic colloids, enzymes, and molecules, increases in viscosity of many fluids, and increased electrolytic dissociation. Probably all of these changes are involved in the biological effects of high pressures, but exact details are obscure.

**Pressure and Temperature.** There is a compensatory relation between pressure and temperature. Unduly high incubation temperatures may be thought of as causing deleterious *expansion* of enzymes (colloids). Increased pressures tend to prevent the expansion and so lessen this unfavorable effect of high temperatures. Cooling may be thought of as causing undue *contraction*, and hence loss of activity, of enzyme colloids. High pressures enhance the retarding effect of low temperatures while relief from pressure tends to overcome the contraction. For example, organisms normally growing best at 20–30° C were completely inhibited at 20° C by 4500 lbs. pressure. They grew well at 40° C under the same pressure.

Marine bacteria are resistant to pressures at the bottom of the sea, which may reach 15,000 lbs. per square inch. Some deep-sea bacteria and others from deep oil wells appear to be favorably influenced by such pressures; they may be said to be *barophilic* (baro = pressure; philic = loving).

**CRUSHING.** Most microorganisms of cellular organization are easily crushed by the impingement upon them of solid particles like steel or glass balls. Shaking in a vessel with steel balls (ballmill) is a method used for the mechanical disruption of bacteria. They shatter especially readily if made brittle by freezing and this method is used for certain chemical investigations of bacteria. Shaking for several hours mixed in a bottle with fine beads of glass accomplishes a complete disruption of the cells.

### SURFACE FORCES

As we have seen, the protoplasm of the living cell is permanently enclosed within a cell wall which mediates many or all of the relationships and communications between the protoplast and the outer world. The exterior surface

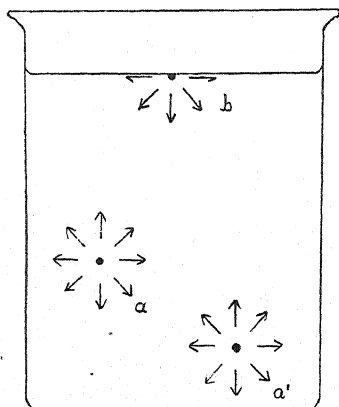


Fig. 12-6. Diagram showing how surface tension acts. The molecules *a* and *a'* are attracted equally from all directions and are in a state of equilibrium. Molecules at the surface, like *b*, are under a greater tension from below than above and the entire surface therefore tends to pull inward much as though the surface film were a rubber membrane.

of the cell wall must, therefore, be especially adapted to carry on various physical and chemical reactions which enable the protoplast to live. In biology, many of the most significant chemical and physical changes, indeed, the entire chemistry of life, take place at, and because of, surfaces. The same forces which act at the outer surfaces of cells also act at the surfaces of the intracellular, protoplasmic, colloidal particles (enzymes). It is evident, therefore, that surface forces are of the most profound importance in all cell life and that we should know something about them.

**Surface Tension.** One of the most important is called *surface tension*, which results from the attraction or cohesive force between molecules. Under its influence the fluid surface acts, in some respects, like an elastic membrane. Many substances, such as soap, bile, certain substances classed as “detergents,” can weaken this intermolecular pull and so lower surface tension. Such substances are called *surface tension reducers*.

**Adsorption.** Of the many phenomena which result from the action of surface tension, probably the most important and best understood is *adsorption*.

Suppose a substance (e.g., soap) which reduces surface tension is added to the water in the beaker shown in Figure 12-6. It must, according to physical laws, tend to carry the reduction of surface tension as far as possible. In this case, the surface tension reductant can lower the surface tension to the greatest extent by accumulating at the surfaces of the fluid. This accumulation is known as *adsorption*. Deposition of the surface tension reductant occurs on the air-fluid surface, at the surface in contact with the glass walls of the containing vessel and on the surface of any particles, such as bacteria, suspended in the fluid.

**ADSORPTION AND RATE OF REACTION.** It has been shown that substances adsorbed upon a surface are often greatly *concentrated* and that, in many cases, their *solubilities increase*. It is probable that some gases are actually liquefied when adsorbed. It is clear that such condensations and increases in solubility must, from the *law of mass action*, greatly facilitate chemical reactions, since the law of mass action states that the greater the concentration of the reacting substances the more rapid the reaction between them. Catalytic

agents (including the enzymes in all living cells), which greatly speed up chemical reactions, depend upon this law.

**Surface Tension and Wetness.** An important result of the action of surface tension reducents is that many important fluids: cell fluids, disinfectants, culture media, paints, cosmetics, and drugs, are really *wetter* than solutions with high surface tension. This wetness brings fluids of low surface tension into more intimate and effective contact with surfaces. Mercury, for example, at room temperatures has a very high surface tension (466 dynes per cm). As a result, as everyone who has held mercury in the palm of the hand knows from experience, mercury possesses the property of wetness in a negligible degree. The inward pull between the molecules at the surface causes liquid mercury to draw inward into globules of itself in a very unsociable manner. It does not spread out and come into intimate contact. Water has a moderately high surface tension (77 dynes per cm) and a moderate degree of wetness, while alcohol has a much lower surface tension (about 28 dynes per cm). Alcohol, therefore, wets or spreads when in contact with surfaces much better than does water and thus comes into more intimate contact with objects which it touches. It is wetter than water: physiologically, politically and socially!

The importance of surface tension and adsorption to the living cell\* can hardly be exaggerated. For example, fission of some species of bacteria growing in media of abnormally low surface tension is delayed so that large spheres and very long filaments and bacilli are formed. Spore formation may be delayed or abolished. Some bacteria will not grow in media having an excessively low surface tension.

A serious difficulty in studies of the effect of low surface tension as a biological factor lies in the difficulty of differentiation between the *physical* effects of a surface tension reducent (low S.T.) and its effects as a *chemical* agent. For example, at the same surface tension, say about 40 dynes per cm, peptone will nourish, phenol will kill.

The actual roles of surface tension and adsorption in microbiology will be referred to more in detail at various places farther on. Many of the substances which nourish living cells are potent surface tension reducents; so are some very important disinfectants. The reactions in the blood and tissues which protect us from disease are all surface reactions involving soluble proteins. All soluble proteins are surface tension reducents. Protoplasm itself has a low surface tension.

### RAPID VIBRATIONS

Bacteria, because of their small mass and their relatively tough cell walls, are not readily disrupted by slow vibration. The "high" limit of audible tone has a vibration rate of around 10,000 per second, the lowest audible tone has a vibration rate of around 100 per second. This is the range of sonic vibrations.†

**Production and Effects of Vibrations.** Vibrations for microbiological purposes are generally produced in fluids by vibrating disks of nickel or quartz crystals, under the influence of alternating electric currents. Sonic or super-

\* and to the student of microbiology!

† Much depends on the intensity and quality of the sound and the individual ear.

sonic waves, to be effective, must be of such pitch that the cells to be disrupted vibrate in harmony with them. The vibrations must also be of considerable intensity.

Immersed in fluid sustaining vibrations of appropriate rate and intensity, bacteria are torn, the protoplasm disrupted, and the cells killed. Much of the damage is believed due to *cavitation*: the formation of a foam of minute bubbles of the gas which is ordinarily in solution in the protoplasm or in the fluids at the bacterial cell surface.

Bacteria differ greatly in their susceptibility to vibration. Some, like the diphtheria bacillus (*Corynebacterium diphtheriae*) appear to be very tough indeed, while the gonococcus (*Neisseria gonorrhoeae*) is fragile and easily ruptured.

Mechanical methods of shattering bacteria (crushing, vibrating, etc.) are of value in experiments in which it is desired to kill and disrupt the organisms without producing such profound chemical and physical changes as result from heating, chemical destruction, exposure to sunlight, or age. Delicate poisons, proteins, carbohydrates or enzymes inside the cells are liberated in an unaltered state by sonic and ultrasonic vibrations and crushing and may thus be studied in an unchanged condition.

#### NATURAL ENVIRONMENTS

Many of the factors influencing microorganisms, which have been discussed in this chapter, occur in Nature in the environment of microorganisms, and many of these factors are, in turn, influenced and altered by neighboring microorganisms. For example, shortages of food, moisture or oxygen; unfavorable pH; concentration of various metallic and other ions, antibiotic substances, etc., may result from the activities of one microorganism near another. On the other hand, one microorganism may create ideal conditions of growth for another, perhaps by synthesizing a vitamin or producing a food substance (e.g.,  $\text{NaNO}_3$ ) by oxidation of its own food substance (e.g.,  $\text{NaNO}_2$ ) (see *Nitrobacter*). If we eliminate these naturally occurring factors, as is generally done in pure culture studies in the laboratory, we obtain a curiously unbalanced knowledge of microorganisms.

Consider, for example, some minute recess a few inches below the surface of a soil rich in organic matter and moisture. Here we find strict aerobes and anaerobes growing side by side decomposing cellulose and proteins to simple substances available to many other soil microorganisms. Fermentation of the cellulose derivatives results in acid production, which may inhibit many bacteria in the neighborhood and may injure crops. Other microorganisms may metabolize the acids, relieving the acid condition of the soil. These and other organisms produce compounds of an endless variety, all available to some part of the soil flora and fauna. The growth of the anaerobes may be favored by the utilization of excess oxygen, or catalase production, by the aerobic bacteria. The fertility of the soil may be increased because autotrophs grow, forming nitrites from ammonia produced from proteins by the proteolytic heterotrophs; and nitrobacter can then grow, oxidizing these same nitrites to nitrates (a plant food). At the same time, all of these bacteria may be held in check and perhaps killed by the poisonous substances poured forth by certain of the *Actinomyces*, molds and *Pseudomonas* group, spore-forming

organisms alone outliving all others. There is constant necessity for adjustment to changing temperatures, osmotic pressures, surface tensions, pH, as well as changing composition of suspending fluid due to fluctuation of water. Such conditions are held fairly constant in most artificial media. Species that cannot adapt themselves to these competitive situations, or form spores, perish.

It is plain that environmental vicissitudes and interactions between various forms of life are the usual experiences of free-living microorganisms, as contrasted with growth in isolated tubes and completely segregated artificial communities (pure cultures) under the most absolutely uniform and favorable conditions possible in the laboratory.

Even those strict parasites which never survive in nature outside the animal or plant body seldom find the site of their entrance into, or growth upon or in, the body devoid of other bacteria. One need only think of the mixtures of organisms in the oral, nasal and intestinal cavities. The science of immunology has taught us something about reactions between pathogenic bacteria and their animal hosts, and we know that each produces alterations in the other. Our knowledge of influence exerted upon one another by microbial species in soil, intestine, milk, etc., is still limited, and so is our knowledge of the effects of those influences. To what extent, for example, can transfer of genetic material by 'phage (*transduction*) occur among bacteria pullulating in rotting organic material? There are high mysteries in a dung heap!

The point is, that laboratory studies generally fall far short of being exact investigations of microorganisms because they do not reproduce the natural environments of the organisms.

#### GERM-FREE LIFE

As we know, microorganisms constantly occur almost everywhere in our environment as well as on all parts of the body surface, the entire alimentary canal, the upper respiratory system, the eyes, the ears, and the genital openings. The newborn animal is contaminated at birth and remains an involuntary and often an unknowing host to billions of various species of microscopic parasites throughout his life. This relationship has existed since archeozoic periods, and animal life is well adapted to existence in contact with most microbial life. Only a few species of microorganisms cause much disturbance in modern man; among them are pathogens like typhoid bacilli, the pneumococcus, the gonococcus, and the tubercle bacillus. It would be of great interest, and extremely useful to know how this age-long contact between animal and microorganism has affected the hereditary, physiological, biochemical, anatomical and other properties of each of the symbionts.

What effect does the enormous number of supposedly harmless bacteria in the intestine have on our nutrition? Do they synthesize vitamins for us? Do they produce antibiotics which are taken into our systems? Do they produce poisons which damage us subtly and shorten our lives, or do they exert favorable or even absolutely necessary influences? Do they immunize us to infection? What is the role of each of the species?

We begin to realize their importance when it is found that suppressing certain groups of bacteria which normally inhabit the gastrointestinal tract (as sometimes occurs during antibiotic therapy) permits other groups, generally

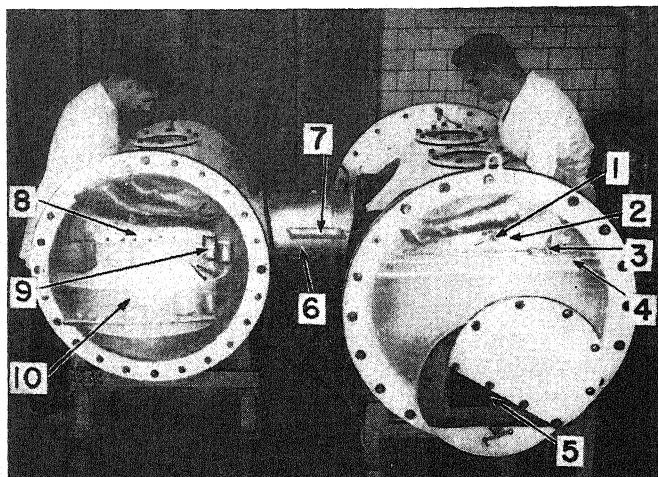


Fig. 12-7. Operating unit (right) connected for aseptic transfer of caesarean-delivered young to germ-free rearing unit (left). Pregnant animal enters at (5), a compartment kept separate from inner operating compartment (2) by a cellophane sheet (4). Animal (1) is brought into close contact with cellophane and young are lifted through it aseptically. (3) Cautery; (6) sterile passage; (7) pan with young; (8, 9, 10) sterile food, supplies, etc.

held in abeyance, to grow, often with evil and sometimes fatal results. Could we answer some of these questions by producing an animal entirely free from microorganisms and then contaminating it with a single species of bacterium at a time?

To do such a thing it would be necessary, by use of fantastically rigorous aseptic technique, to separate an animal from all demonstrable microorganisms at the beginning of life, to maintain it for months or years free from any demonstrable live microorganisms, and then to observe how it fares in life free from its usual living mates, the microorganisms. Such an animal might be said to represent "germ-free" life, or axenic life (*a* = free from; *xenic* = foreign substance), or gnotobiotic life (*gnote* = known or exactly defined; *bios* = life.)

Pasteur realized the value of such investigations as early as 1885. Due to the great difficulties involved, only four workers prior to 1928 had attempted to rear germ-free animals. In 1928 Reyniers started work on chickens and, with his co-workers, has succeeded in raising numerous germ-free animals. Completely germ-free insects, fish, chickens, rodents, dogs, pigs and monkeys have been born and held germ-free for many months. Second generations of white rats and chickens have been reared completely devoid of any living microorganisms.

The very expensive apparatus for such work consists of large steel cylinders which can be steam sterilized; with closed, glass observation ports; hand-holes fitted with air-tight, arm-length, seamless, rubber gloves; and air-tight systems of outer chambers or locks through which sterilized food, water and equipment are passed into the chamber while waste materials are passed out. Air is passed through sterilizing filters and conditioning apparatus (Fig. 12-7). Such equipment and its operation have necessitated solution of some difficult engineering problems. One of the newest chambers is big enough for an attendant to enter, dressed in a diving suit, through a deep tank of disinfectant (Fig. 12-8). Careful

control is necessary at all times, and all animals and their feces, and bodies, and the dust, feed, water, etc., in the germ-free compartments are examined bacteriologically at short intervals to detect any contamination.

Some of the technical difficulties are very great, as for example, feeding young rats delivered aseptically at Caesarean section. They require milk about every hour, 24 hours a day, for weeks on end. It took much research to synthesize a satisfactory substitute for mother rat's milk! These studies on germ-free life have been carried on at the Laboratories of Bacteriology at the University of Notre Dame (Lobund).

The germ-free animals in general live longer and seem healthier than ordinary animals. However, they are often very susceptible to fatal infections with many, usually-harmless bacteria. The living cells of germ-free rats and chickens seem to remain "younger" than those of "normal," contaminated animals. Is this because they are germ-free? If antibiotics are fed to farm stock, this possibly reduces their bacterial burden. They certainly grow much faster.

A very important observation was made when it was found that germ-free guinea pigs *did not develop dysentery* when infected with the ameba, *Entamoeba histolytica*, which usually causes a particularly bad sort of dysentery in guinea pigs. Now, it is known that *E. histolytica* feeds on bacteria. In germ-free guinea pigs there were *no bacteria*. The infected germ-free guinea pig, *free from disease*, quickly developed dysentery when fed ordinary "harmless" intestinal bacteria and died! It is suggested that certain antibiotics are effective in curing amebic dysentery not because they effect amebae but because they deprive the amebae of their food, the bacteria.

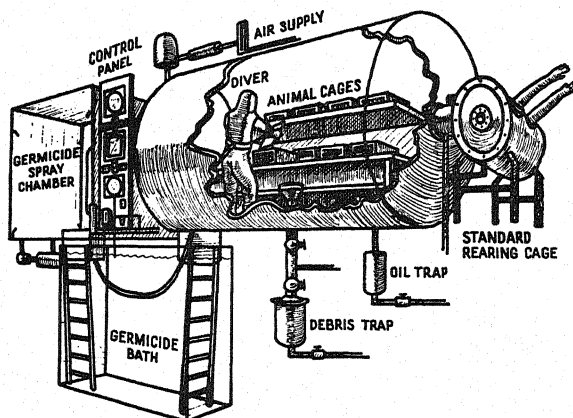


Fig. 12-8. Arrangement of germ-free animal quarters. At right is the standard rearing cage, with rubber arm-gloves, and sterile lock opening into main compartment. The traps beneath are for removal of waste without back-flow of contaminated material; overhead, a sterile air supply. The operator in air-tight dress enters the main chamber through a spray and tank of strong disinfectant, dragging an air hose like a diver. Air conditioning is electrically automatic. (Figures 12-7 and 12-8 courtesy of Dr. James A. Reyniers, University of Notre Dame, Notre Dame, Ind., 1950.)



## REFERENCES

- Abelson, P. H., and Aldous, E.: Ion antagonisms in microorganisms. *J. Bact.*, 1950, 60:401.
- Bates, R. G.: *Electrometric pH Determinations*. John Wiley and Sons, Inc., New York, 1954.
- Bawden, F. C., and Kleczkowski, A.: Studies on the ability of light to counteract the inactivating action of ultraviolet radiation on plant viruses. *J. Gen. Micr.*, 1955, 13:370.
- Bellamy, W. D., and Germain, M. T.: An attempt to photoreactivate ultraviolet inactivated streptococci. *J. Bact.*, 1955, 70:351.
- Bellamy, W. D., Goldblith, S. A., Colovos, G. C., Niven, C. F., Jr.: Symposium on applications of ionizing radiation to food and pharmaceutical preservation. *Bact. Rev.*, 1955, 19:266.
- Burkholder, P. R.: Cooperation and conflict among primitive organisms. *Am. Sci.*, 1952, 40:601.
- Ellison, S. A., Erlanger, B. F., and Allen, P.: The chemical reversal of ultraviolet effects on bacteria. *J. Bact.*, 1955, 69:536.
- Flannery, W. L.: Current status of knowledge of halophilic bacteria. *Bact. Rev.*, 1956, 20:49.
- Frobisher, M., Jr., Klein, G. C., and Cummings, M. M.: Preservation of mycobacteria by desiccation in vacuo. *Am. Rev. Tuberc.*, 1949, 60:621.
- Gary, N. D., Kupferberg, L. L., and Graf, L. H.: Demonstration of an iron-activated aldolase in sonic extracts of *Brucella suis*. *J. Bact.*, 1955, 69:478.
- Gerhardt, P., Vennes, J. W., and Britt, E. M.: Gram reaction of isolated protoplasts and surface membranes of *Bacillus megaterium*. *J. Bact.*, 1956, 72:721.
- Glassman, H. N.: Surface active agents and their application in bacteriology. *Bact. Rev.*, 1948, 12:105.
- Goucher, C. R., Sarachek, A., Kocholaty, W.: A time-course respiratory inactivation associated with *Azotobacter* cells deprived of  $Mg^{++}$ . *J. Bact.*, 1955, 70:120.
- Gwatkin, R. B., and Gottlieb, D.: Mutants recovered after exposure of *Streptomyces venezuelae* to x-rays. *J. Bact.*, 1956, 71:328.
- Harden, V. P., and Harris, J. O.: The isoelectric point of bacterial cells. *J. Bact.*, 1953, 65:198.
- Harris, R. J. C., Editor: *Biological Applications of Freezing and Drying*, Academic Press, New York, 1954.
- Harris, R. J. C., Greaves, R. I. N., Annear, D. I., Bell, L. G. E., and Vallet, L.: *Freeze-Drying*. Lab. Pract. (London), 1956, 5:5, 53, 102, 139, 169.
- Hartman, R. S., et al.: An electrophoretic method for the assay of bacterial variants. *Appl. Micr.*, 1953, 1:178.
- Hollaender, A., Editor: *Radiation Biology*, I, II, III. McGraw-Hill Book Co., New York, 1956.
- Hugo, W. B.: The preparation of cell-free enzymes from microorganisms. *Bact. Rev.*, 1956, 18:87.
- Kasai, G. J.: Growth response of microorganisms to vitamins at different temperatures. *J. Inf. Dis.*, 1953, 92:58.
- Kelner, A., Bellamy, W. D., Stapleton, G. E., and Zelle, M. R.: Symposium on radiation effects on cells and bacteria. *Bact. Rev.*, 1955, 19:22.
- Kempe, L. L., Graikoski, J. T., and Gillies, R. A.: Gamma ray sterilization of canned meat previously inoculated with anaerobic bacterial spores. *Appl. Micr.*, 1955, 3:330.
- Kinsloe, H., Ackerman, E., and Reid, J. J.: Exposure of microorganisms to measured sound fields. *J. Bact.*, 1954, 68:373.
- Koh, W. Y., Morehouse, C. T., and Chandler, V. L.: Relative resistance of microorganisms to cathode rays. I, II, III. *Appl. Micr.*, 1956, 4:143, 147, 149.
- Kushner, L. M., and Hoffman, J. I.: Synthetic detergents. *Sci. Am.*, 1951, 185:26.
- Lamanna, C., and Mallette, M. F.: *Basic Bacteriology*. Williams & Wilkins Co., Baltimore, 1953.
- Loebeck, M. E., and Klein, H. P.: Substrates for *Myxococcus virescens* with special reference to eubacterial fractions. *J. Gen. Micr.*, 1956, 14:281.
- Oginsky, E. L., and Rumbaugh, H. L.: A cobalt-activated bacterial pyrophosphatase. *J. Bact.*, 1955, 70:92.
- Oginsky, E. L., and Umbreit, W. W.: *An Introduction to Bacterial Physiology*. W. H. Freeman and Co., San Francisco, Calif., 1954.

- Parkes, A. S.: The freezing of living cells. *Sci. Am.*, 1956, 194:105.
- Perlman, D., et al.: Symposium on the maintenance of cultures of microorganisms. *Bact. Rev.*, 1955, 19:280.
- Phillips, B. P., Wolfe, P. A., Rees, C. W., Gordon, H. A., Wright, W. H., and Reyniers, J. A.: Studies on the ameba-bacteria relationship in amebiasis. *Am. J. Trop. Med. & Hyg.*, 1955, 4:675.
- Reyniers, J. A.: Some observations on rearing laboratory vertebrates germ-free. *Proc. New York State Assoc. of Pub. Health Labs.*, 1949, 28:60.
- Reyniers, J. A., Trexler, P. C., Erwin, R. F., Wagner, M., Luckey, T. D., and Gordon, H. A.: Germ-free life studies. *Lobund Reports*, 1946, No. 1, No. 2, 1949.
- Rochford, E. J., and Mandle, R. J.: The production of chains by *Diplococcus pneumoniae* in magnesium deficient media. *J. Bact.*, 1953, 66:554.
- Rountree, P. M.: The role of divalent cations in the multiplication of staphylococcal bacteriophages. *J. Gen. Micro.*, 1955, 12:275.
- Sachs, M., and Sullivan, R.: Radiological health or radiation protection? *Am. J. Pub. Health*, 1956, 46:575.
- Shankar, K., and Bard, R. C.: Effect of metallic ions on the growth, morphology, and metabolism of *Clostridium perfringens*. *J. Bact.*, 1955, 69:436.
- Skinner, F. A.: Inhibitions of the growth of fungi by *Streptomyces* spp. in relation to nutrient conditions. *J. Gen. Micro.*, 1956, 14:381.
- Stevenson, I. L.: Antibiotic activity of Actinomycetes in soil and their controlling effects on root rot of wheat. *J. Gen. Micro.*, 1956, 14:440.
- Symposium: Survival of Dried Cultures. A Discussion on the Maintenance of Cultures by Freeze-drying. Her Majesty's Stationery Off., Code No. 88-1311. London, 1954.
- Talmage, D. W.: Effect of ionizing radiation on resistance and infection. *Ann. Re. Microbiol.*, 1955, 9:335.
- Tarpley, W., Ilavsky, J., Manowitz, B., and Horrigan, R. V.: Radiation sterilization. *J. Bact.*, 1953, 65:305.
- Wainwright, S. D., and Nevill, A.: Modification of the biological effects of ultraviolet irradiation by post-irradiation treatment with iodoacetate and peptone. *J. Gen. Micro.*, 1955, 12:1.
- Weaver, W. (Chairman): Report of Committee on genetic effects of atomic radiation. *Science*, 1956, 123:1157.
- Weinberg, E. D.: The effect of  $Mn^{++}$  and antimicrobial drugs on sporulation of *Bacillus subtilis* in nutrient broth. *J. Bact.*, 1955, 70:289.
- Wellerson, R., Jr., and Tetrault, P. A.: The effect of various incubation temperatures on the ribonucleic acid production of a mesophilic and thermophilic bacterium. *J. Bact.*, 1955, 69:449.
- Welsh, J. N., and Adams, M. H.: Photodynamic inactivation of bacteriophage. *J. Bact.*, 1954, 68:122.
- Werkman, C. H., and Wilson, P. W.: *Bacterial Physiology*. Academic Press, New York, 1951.
- ZoBell, C. E.: *Marine Microbiology*. The Chronica Botanica Co., Waltham, Mass., 1946.
- ZoBell, C. E., and Oppenheimer, C. H.: Some effects of hydrostatic pressure on the multiplication and morphology of marine bacteria. *J. Bact.*, 1950, 60:771.

## Nutrition, Metabolism, and Cultivation of Microorganisms

### NUTRITION

**Food.** Napoleon is reputed to have said, "An army marches on its stomach." In establishing this military aphorism he missed the opportunity to become a biological immortal. He could have stated the obvious truth that all life depends on food. To Bonaparte, food meant army rations. However, the word means various things as indicated by the cliché, "one man's food is another man's poison." Here we shall mean, by food, any substance which, taken into a living organism, yields energy to that organism in a physiologically useful and beneficial manner and/or furnishes material by means of which the organism may synthesize itself, either directly or after modifying the food by digestive and/or other processes to fit the physiological requirements of the organism. The reader may at first be inclined to consult a lawyer in attempting to interpret this definition. However, it is hoped that a perusal of the following paragraphs will obviate this necessity.

**Holozoic Nutrition.** Most biologists will agree that not only soldiers but all *animals*, from man to protozoa, with few exceptions have the ability to take *solid* food materials into the organism. After being ingested the food is first digested by hydrolytic enzymes. This takes place in some form of gastrointestinal tract (of larger animals) or intracellular food-digestion vacuole (of animal cells like protozoa).

If the food is a complex, organic material such as protein, fat or carbohydrate digestion results in its being enzymatically decomposed into its constituent molecules of various soluble substances such as amino acids, fatty acids and glucose. Unlike the original solid food mass, these molecules are small enough to pass from the gastrointestinal tract (or digestive vacuole) *through the cell wall* into the living protoplast. This mode of nutrition is characteristic of the animal kingdom and is said to be *holozoic*.

Virtually all complex foodstuffs are reduced by digestion to the common denominator of relatively small, simple and soluble molecules before they can be used as nutrients\* in any cell. Once inside the protoplast proper they are used either in cell synthesis or as sources of energy.

\* The term *nutrient* is often used as though it were synonymous with food, and indeed it may be. Food is probably a more inclusive term. We shall use nutrient here to refer to *soluble* substances capable of furnishing energy or cell substance which pass through the cell

**Holophytic Nutrition.** Unlike animals, plants, including bacteria, yeasts, molds (and, probably, rickettsiae, PPLO and viruses), have no such "built-in" digestive mechanisms as those described above. The digestive mechanisms of plant cells are obviously less well organized than are animal digestive mechanisms. Plants\* cannot take *solid* food particles into the organism. All of their food must be in aqueous solution. This sort of nutrition, characteristic of plants, is said to be *holophytic*.†

Many species of bacteria, yeasts and molds can, nevertheless, use the same, solid foods that soldiers, horses and protozoa enjoy. This they do by what has been called "extracorporeal digestion."‡

Bacterial digestive enzymes, for example, are not concentrated in a gastrointestinal tract or vacuole but are, to a large extent, excreted aimlessly into the surrounding fluid where they may or may not come into contact with food. They may be wholly dissipated by dilution, convection currents, or other factors. Assuming that the digestive enzymes come into contact with digestible food, the food is decomposed (very much as occurs in the animal digestive tract) into the same sorts of relatively small, simple, soluble molecules as result from animal digestive processes. As in animal nutrition, these molecules pass through the bacterial cell wall into the protoplast.

From this point on the processes of food utilization (*metabolism*) in plant and animal cells (including the cells of our own bodies) are astonishingly alike though by no means identical. They are all obviously modifications of the same fundamental plan. Yet each species of plant and animal in this respect differs to some degree from every other species of plant and animal. A thousand different tunes may be made using only eight notes of the diatonic scale.

In spite of their hit-or-miss digestive systems, an interesting advantage possessed by many species of yeasts, molds, bacteria and related microorganisms over animals is their indifference in matters of taste. For example, a species of bacterium or mold, given time and numbers (and if it possesses the proper enzymic equipment) may use as food with equal avidity a railroad tie, crab shells, a dead horse's hoof, a defunct cow's horn, feces, paper, leather, crude transmission grease, sawdust, old rubber tires, and so on. Digestion of the solid organic food stuff is accomplished outside of the cell. All that the cell takes in are the soluble products of the digestive process: glucose, amino acids, fatty acids, vitamins, minerals, and so on. The cell is indifferent to their source. It never has indigestion or ulcers!

Few if any bacteria or molds are actually so very versatile in their ability

\* Except a few curious carnivorous plants like Venus's fly trap and the pitcher plant.

† If we consider the *tissue cells* of large animals, even this difference largely disappears, because the tissue cells depend on food in solution in the blood stream.

‡ Digestion which takes place *outside the body proper*.

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wall or which occur temporarily in the cell during metabolism. This would include not only simple carbohydrates and related compounds (glucose, alcohols, and the like) simple nitrogenous compounds (amino acids) fatty acids, minerals, vitamins and related substances derived from food but various temporary, intermediate products of metabolic processes which take place in the cell. Glucose, for example, could be regarded as a nutrient or as a food. But starch, which is not metabolized per se but from which glucose is derived by digestion, is a food.

to use *all* of the varied foods indicated above but some approach this degree of versatility, and all of the substances mentioned, plus hundreds of others equally distasteful or poisonous to us, are food for one or other species of microorganisms or combinations of them acting together. In contrast, other microorganisms are highly restricted and fastidious in the matter of food and can thrive only upon certain particular compounds. Examples of the various kinds of microorganisms will be described farther on.

#### NUTRITIONAL TYPES

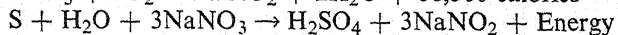
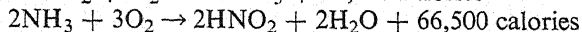
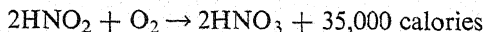
**General Requirements.** While plant and animal species differ somewhat in the details of their various food requirements, we may note one universal necessity: water. The presence of water in adequate amounts will be assumed in all discussions of nutrition and metabolism which follow.

**Elemental Requirements.** The evolution of microorganisms (represented here by bacteria) from the most primitive forms of life, and their kinship with all other forms of life from man to virus, are revealed by the universal, basic, protoplasmic requirement for certain elements: C, H, O, N, P, K, Na, S, Fe, Mg, Ca, and various others. Protoplasm is made up of compounds containing these elements. To serve as nutrients for most microorganisms they must be in forms soluble in water and capable of passing through the cell wall and membranes (*assimilable forms*).

The exact list of elemental requirements for living cells is not fully known since some elements are required in such minute amounts (*trace elements*) that it is very difficult to detect them by analytical methods. The requirements may vary slightly from species to species but are pretty much the same for all.

**Autotrophs.** Some bacteria require as food *nothing* but a few, simple, inorganic substances such as NaCl, K<sub>2</sub>HPO<sub>4</sub>, FeCl<sub>3</sub>, MgSO<sub>4</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to live and multiply. They obtain their carbon from atmospheric CO<sub>2</sub> or from carbonates. Such organisms are said to be *autotrophic*.<sup>\*</sup> From such simple inorganic materials autotrophic microorganisms synthesize their complex chemical structures: proteins, fats, carbohydrates, vitamins, enzymes, cell walls, genes, cytoplasm and so on.

✓ **CHEMOSYNTHETIC AUTOTROPHS.** The primeval earth is believed to have been completely dark due to heavy clouds of vapor in the skies. It may (or may not!†) be due to this fact that some primitive organisms, such as the autotrophic bacteria, do not depend on sunlight as their source of energy as do familiar green plants. They obtain the energy for their life processes from chemical reactions involving oxidations of inorganic materials such as H<sub>2</sub>S, H<sub>2</sub>NO<sub>2</sub>, and NH<sub>4</sub>OH. For example:



Because they obtain the energy to synthesize themselves from chemical reactions they are said to be *chemosynthetic*.

<sup>\*</sup> *Auto* is from a Greek word meaning self; *trophic* is from a Greek word meaning nourishing.

† You may argue on either side with equal profit!

**PHOTOSYNTHETIC AUTOTROPHS.** A few species of autotrophic bacteria, characteristically occurring in sewage-polluted sea and brackish waters, possess the ability to derive energy from the sun much as do green plants. They contain chlorophyll-like, photosynthetic pigments. These alga-like species are discussed more fully in a later chapter (Chapt. 29). They may have evolved from chemosynthetic autotrophs after sunlight appeared on earth.

**Heterotrophs.** There is good reason to believe that some of the earliest forms of life on earth developed in primeval oceans, lakes and muds in close association with simple, spontaneously-occurring\* organic compounds such as aldehydes, glucose, ammonium carbonate, urea, amino acids, etc. From such compounds very complex organic structures may be built up by polymerizations, simple conjugations, etc. Indeed, the earliest forms of life (much simpler than bacteria) probably evolved from such self-initiating processes. Be that as it may, microorganisms eventually developed which utilized such compounds as foods. Their descendants today are characterized by *absolutely requiring* at least one organic compound as a source of energy. Such microorganisms are said to be *heterotrophic*.†

Glucose is probably most commonly used as an energy source by heterotrophs, but is not necessarily the only one.

Many heterotrophs have the ability (some absolutely require) to use CO<sub>2</sub> as cell building material, at least for part of their needs. This CO<sub>2</sub> requirement of heterotrophs may represent a vestigial inheritance from an autotrophic ancestry of millions of years ago.

Heterotrophs are by far the commonest, most widely distributed, and numerous types among microorganisms.

**CHEMOSYNTHETIC HETEROTROPHS.** It seems likely that the early heterotrophic microorganisms developed under conditions of darkness similar to those surrounding the development of chemosynthetic autotrophs. Thus, the commonest forms of heterotrophic microorganisms are chemosynthetic. They derive their energy only from oxidations of *organic* compounds.

**PHOTOSYNTHETIC HETEROTROPHS.** We have noted that among the autotrophic bacteria there are a few species which have developed the ability to utilize sunlight as a source of energy by means of photosynthetic pigments resembling the chlorophyll of green plants. Similarly, some species of heterotrophs have developed the same form of metabolism. These curious photosynthetic bacteria, both autotrophic and heterotrophic, are discussed in Chapter 29.

**Saprophytes.** Most of the earliest heterotrophic bacteria, yeasts and molds lived entirely upon inert organic compounds; either those spontaneously occurring around them or available from the wastes and dead remains of other organisms. Such heterotrophic forms are today commonplace and are to be found very usefully engaged in sewage-disposal plants, the soil, and in every situation where decomposition and decay of inert, waste and dead

\* Several kinds of organic compounds such as aldehydes, glucose, amino acids and the like have been shown to form in appropriate solutions under presumably primeval, geological conditions without the intermediation of any living thing, enzyme or other organic formative mechanism; i.e., spontaneously.

† From two Greek words meaning *nourished by others*. This refers to the fact that organic foods are generally derived from the wastes or substance of other living things.

organic matter are going forward. They are collectively spoken of as *saprophytes* (from the Greek roots *sapro*, meaning decay, and *phyte*, meaning plant). Most of the bacteria, yeasts and molds known today are of this type.

**Parasites.** Probably still later there appeared, either through progressive or regressive evolutionary stages or both, bacteria, etc., which could live not only upon dead and waste organic matter, but which could also live in living plant and animal tissues. They caused disturbances of the delicate chemical and physical equilibria of the creatures in which they lived. This was disease and often resulted in the death of the invaded creatures. Such organisms are well known today and are said to be *parasitic*\* or *pathogenic*.\*

### RECAPITULATION

We may simplify the relationships of these various groups of microorganisms by tabulating them as follows:

- I. **Autotrophs.** Require inorganic food only.
  - A. *Chemosynthetic*: obtain energy only from chemical reactions (oxidations); no photosynthetic pigments.
  - B. *Photosynthetic*: obtain energy from sunlight; contain chlorophyll-like pigments (alga-like).
- II. **Heterotrophs.** Require organic source of carbon and energy.
  - A. *Chemosynthetic*
    1. Saprophytes: as a rule live entirely on inert organic matter; not ordinarily involved in production of disease.
    2. Parasites or pathogens: may or may not be able to live as saprophytes but can and do live in, or upon, other living organisms, causing disease.
  - B. *Photosynthetic*: obtain energy from sunlight; contain chlorophyll-like pigments (alga-like).

### NUTRITIONAL REQUIREMENTS AND CULTURE MEDIA

In order to *cultivate* microorganisms, i.e., to induce them to grow and multiply under laboratory or other specified conditions it is necessary to know not only about the distinguishing requirements of the major nutritional groups but the actual, specific, food requirements of each family, genus or species which it is desired to cultivate. Any *culture medium*, i.e., any nutrient fluid or material in (or on) which we expect to cultivate living organisms: man, fish or bacteria, must contain: (1) sources of energy; (2) sources of cell-building material; and (3) for many forms other than autotrophs, certain accessory substances which do not yield energy or any significant amount of cell substance but which are absolutely essential to normal growth. These are exemplified by vitamins, various amino acids, certain minerals or the ions derived therefrom, and the like. The result of dissimilation is a yield of energy to the cell.

✓The utilization of food by living organisms for such purposes is called *metabolism*.

### METABOLISM

#### 1. NUTRIENTS AS SOURCES OF ENERGY

The intracellular reactions involved in the utilization of nutrients as sources of energy are all of the *exothermic* or energy-yielding type. Such processes are

\* (*Parasitic* is from Greek roots signifying to eat the food of another; *pathogenic* is from Greek roots meaning to produce disease).

often designated by the terms *biological oxidation* or *respiration*. These reactions result in the *dissimilation*\* of the nutrient.

Basically, all respiratory processes are modifications of only two types of chemical change: (a) oxidation by *dehydrogenation*; (b) oxidation by oxygen uptake. It is doubted by some that the latter is a source of energy in microorganisms. Both result in the same thing: a yield, to the cell enzyme systems, of energy as a result of the transfer of electrons. Electron transfer in this manner is the basis of all life. The two kinds of respiratory process may be described briefly as follows.

**Dehydrogenation.** This is accomplished by the enzymic removal of hydrogen from a nutrient molecule (or *substrate*) containing it. The particular enzymes involved are called *dehydrogenases*. The substrate being robbed of its hydrogen (oxidized) is called a *hydrogen donor* because it yields up its hydrogen to the dehydrogenase, which is thus reduced. The reduced dehydrogenase passes the hydrogen on to another molecule in the cell called a *hydrogen acceptor* and is thus ready to take up more substrate hydrogen. This process goes on continuously during life.

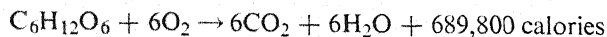
It is worth noting at this point that the actual transfer of the hydrogen is mediated by a part of the dehydrogenase called its *coenzyme*. The part of the coenzyme actually combining with, and passing on, the substrate hydrogen is the nicotinic acid ("niacin") molecule contained in the coenzyme. (See Fig. 13-1.) Thus the role of at least one vitamin in metabolism is clear. It is representative of most others.

In the cell the hydrogen from the substrate is transferred from one hydrogen acceptor molecule to another in a systematic sequence until the hydrogen is at last transferred to some final hydrogen acceptor outside of the cell. As we shall see later, the nature of the final hydrogen acceptor is determined by the enzymic equipment of the cell and is a very constant and distinctive property of various types of cell.

When hydrogen is thus removed from a substrate molecule, an electron is transferred at the same time. Energy is thus released from the substrate and becomes available to the cell. This energy is immediately taken up into the cell by means of a complex system of enzymes. It is absorbed into a particular sort of organic phosphate bond (high-energy phosphate) and is later used as life energy. Thus, the net result of dehydrogenation is decomposition of the substrate and transfer of its energy to the living cell.

✓ **Aerobic Respiration.** If air is present (*aerobic* conditions) the final hydrogen acceptor may be oxygen.  $\text{H}_2\text{O}$  or  $\text{H}_2\text{O}_2$  may be formed from the hydrogen, depending on the species of organism and its enzyme system. *Aerobic* bacteria are generally equipped with the enzyme *cytochrome oxidase* which enables them to utilize atmospheric oxygen as a final hydrogen acceptor.

**COMPLETE AND INCOMPLETE OXIDATION.** The respiratory oxidation of the substrate may be complete, as illustrated by the aerobic utilization of glucose by baker's yeast (*Saccharomyces cerevisiae*) and by many heterotrophic bacteria:



\* In dissimilation the nutrient is decomposed, oxidized, and the products excreted as wastes. Dissimilation is the opposite of *assimilation*, in which the nutrient is absorbed, stored, and eventually built into the cell substance. Parts of some nutrient molecules, for example glucose, are dissimilated; other parts of the same molecule are assimilated.



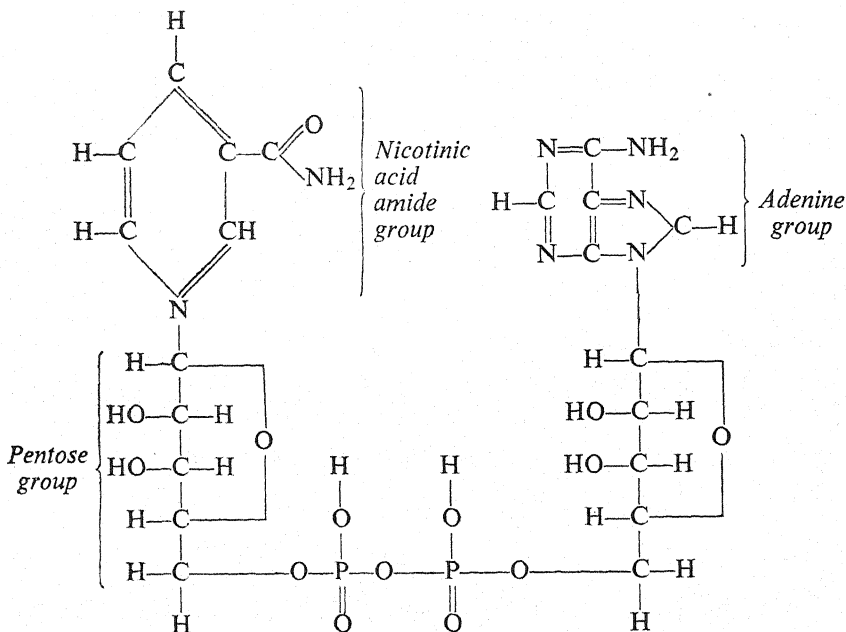
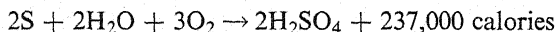


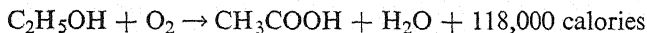
Fig. 13-1. Diagram of the structure of the molecule of coenzyme I. At upper left is seen the nicotinic acid amide ("niacin" vitamin). This is connected with a carbohydrate (pentose) group and this, in turn, with two phosphate groups. At the right these are connected to another pentose group and this, at upper right, to an adenine group. The intricate synthesis necessary to the construction of such a molecule is suggested. It can readily be understood why, if an organism cannot synthesize nicotinic acid amide (as is the case with us poor humans) it must have the vitamin fed to it.

or by the oxidation of sulfur by certain autotrophs:



Such oxidations release all of the energy available from the substrate.

However, the oxidation may be incomplete. Much depends on the species and the enzymic equipment of the cell involved. As an example, the production of acetic acid by the oxidation of alcohol by *Acetobacter* may be noted:



Much of the available energy of the alcohol is left in the acetic acid. An example of the incomplete oxidation of an inorganic substrate ( $\text{NH}_3$  to  $\text{HNO}_2$ ) has previously been given.

**STRICT AEROBES.** A number of common species of microorganisms possess respiratory mechanisms capable of utilizing only free oxygen as final hydrogen acceptor. These must have free access to air for growth. They are called *strict* or *obligate aerobes*.

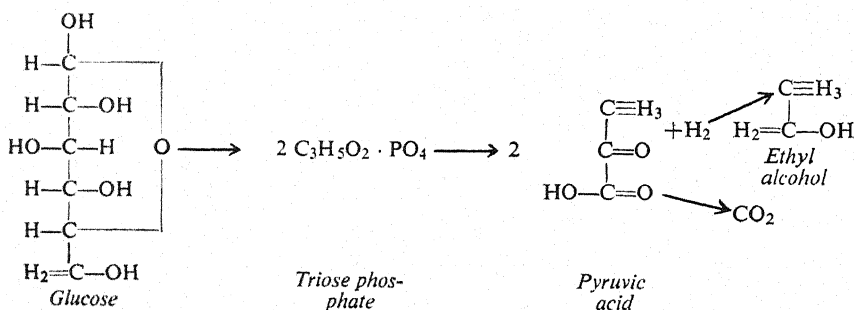
**Anaerobic Respiration.** Many species of microorganism are *anaerobic*. That is, they do not possess the cytochrome oxidase enzyme which would enable them to utilize atmospheric oxygen as a final hydrogen acceptor.

**STRICT ANAEROBES.** As we shall see later, some anaerobes not only cannot utilize, but are actually poisoned by, atmospheric oxygen. These are called *strict* or *obligate* anaerobes and they must be cultivated in the complete absence of air.

When air is not present *anaerobic* conditions are said to exist. Under these conditions microorganisms must obviously use some final hydrogen acceptor other than atmospheric oxygen. This is usually some compound which readily yields oxygen or combines with hydrogen. For example,  $\text{NaNO}_3$  serves as an effective final hydrogen acceptor under anaerobic conditions because it readily yields up oxygen to be combined with the substrate hydrogen. It is reduced to  $\text{NaNO}_2$  in the process. Many bacteria can reduce nitrates to nitrites and the property is commonly tested for in identifying bacterial species. Some other final hydrogen acceptors used by anaerobes are  $\text{H}_2\text{SO}_4$  (reduced to S or  $\text{H}_2\text{S}$ ) and  $\text{CO}_2$  (reduced to  $\text{CH}_4$ ). Others are used, depending on the species of cell involved and its enzymic equipment.

**INTERMOLECULAR RESPIRATION.** Respiration in which the final hydrogen acceptor is some extraneous substance like  $\text{NaNO}_3$ , or possibly some organic compound, is spoken of as *intermolecular respiration* because the hydrogen is transferred from one molecule (substrate) to another.

**INTRAMOLECULAR RESPIRATION.** This is often called *fermentation*. It is the most characteristic, best known and commonest type of anaerobic respiration. It differs from intermolecular respiration mainly in that the final hydrogen acceptor is derived from the nutrient substrate itself. Part of the nutrient molecule is oxidized, part is reduced. For example, in the fermentation of glucose, the 6-carbon sugar (hexose) molecule (which is diagrammatically shown here):



is split by enzymes into two 3-carbon sugars and phosphorylated (triose phosphate).<sup>\*</sup> From such triose molecules pyruvic acid is eventually formed after a series of chemical transformations outlined in the Meyerhof-Embden scheme (see Fig. 13-2). It is evident that, in the process of dissimilation of the glucose, hydrogen has been removed from parts of the molecule (resulting in oxidation) and shifted to other parts of the same molecule (resulting in reduction).

Part of the pyruvic acid is eventually changed to alcohol and  $\text{CO}_2$  as shown above. The net result of this oxidation-reduction process is a release of energy

<sup>\*</sup> As previously noted, the phosphate is involved in the energy transfer.

THE MEYERHOF-EMBDEN SCHEME FOR DISSIMILATION  
OF GLUCOSE TO PYRUVIC ACID

1. Glucose + ATP  $\longrightarrow$  glucose-6-phosphate + ADP  
(hexokinase)
  2. Glucose-6-phosphate  $\rightleftharpoons$  fructose-6-phosphate  
(phosphohexoisomerase)
  3. Fructose-6-phosphate + ATP  $\longrightarrow$  fructose-1,6-diphosphate + ADP
  4. Fructose-1,6-diphosphate  $\rightleftharpoons$  3-phosphoglyceraldehyde + dihydroxyacetonephosphate  
(aldolase)
- $\xrightarrow{\text{(isomerase)}}$
5. 3-Phosphoglyceraldehyde +  $\text{H}_3\text{PO}_4 \rightleftharpoons$  (1,3-diphosphoglyceraldehyde)
  6. (1,3-Diphosphoglyceraldehyde) + DPN  $\rightleftharpoons$  1,3-diphosphoglyceric acid +  $\text{DPNH}_2$
  7. 1,3-Diphosphoglyceric acid + ADP  $\rightleftharpoons$  3-phosphoglyceric acid + ATP
  8. 3-Phosphoglyceric acid  $\rightleftharpoons$  2-phosphoglyceric acid
  9. 2-Phosphoglyceric acid  $\rightleftharpoons$  phosphoenol pyruvic acid +  $\text{H}_2\text{O}$
  10. Phosphoenol pyruvic acid + ADP  $\rightleftharpoons$  pyruvic acid + ATP

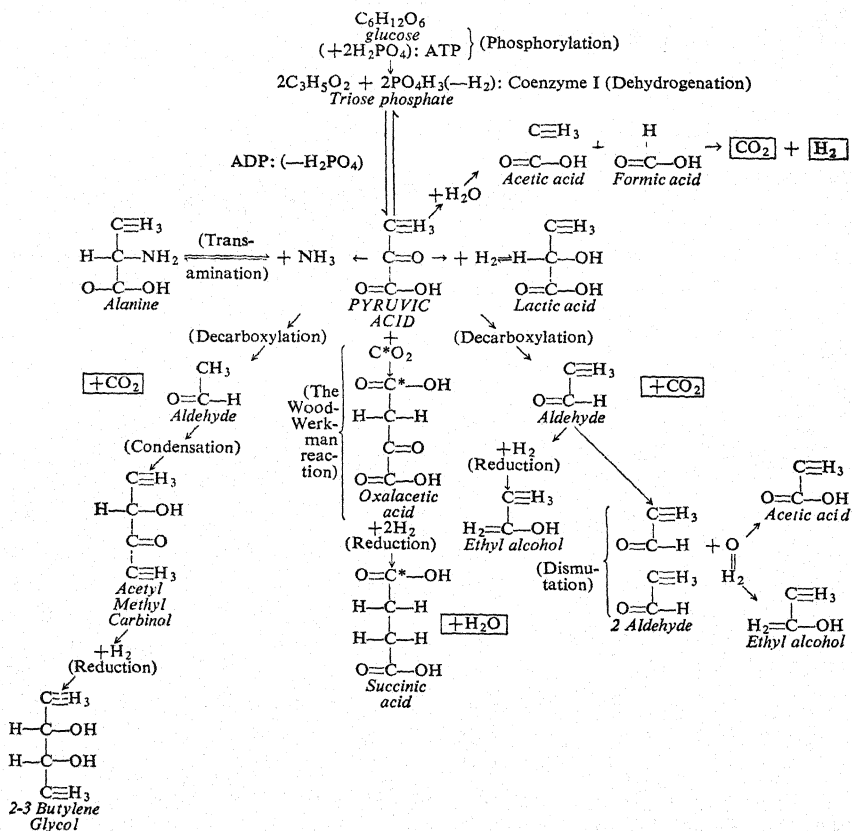


Fig. 13-2. Common metabolic pathways illustrated by the dissimilation of glucose by *Klebsiella aerogenes*. This is a "chemical map," indicating successions of reactions; it is in no sense a balanced equation. Pyruvic acid is seen as a sort of biochemical turn-table, reached through a set of transformations of glucose involving dehydrogenation, phosphorylation, etc., as described in the Meyerhof-Emden scheme and text. Pyruvic acid may under go one or more of several changes, the end products of which are shown in italics. Types of-chemical change are in parentheses. Note that amino acids, represented by alanine, can enter this system as well as glucose. Hydrogen, carbon dioxide and water, as wastes, are seen in square boxes. Not all of the pathways are fully agreed upon, but most of them are. The end-products are all well established.

from the glucose molecule to the cell. Note that pyruvic acid itself (after decarboxylation) acts as a hydrogen acceptor in being reduced to alcohol.

**RESULTS OF FERMENTATION.** Fermentation in general results in incomplete oxidation. Depending on the species of organism, the substrate and the cultural conditions, some of the products of fermentation of glucose may be butyl alcohol, acetone, lactic and acetic acids, and so on. Some of these are of great industrial value and will be mentioned later. The fermentation of glucose by yeast, a common industrial process, results in the formation of alcohol and  $\text{CO}_2$  as described above (Meyerhof-Embden scheme):



Compare this with the aerobic dissimilation of glucose given previously.

**Stages and Products of Energy Metabolism.** For those readers who have had considerable organic chemistry there are here given in outline the Meyerhof-Embden scheme showing the chemical stages in the dissimilation of glucose by yeasts and (often in modified form) by many other microorganisms. These readers may also be interested in the diagrammatic illustration of some of the terminal dissimilative changes following the formation of pyruvic acid.

**DISTINCTIVE METABOLIC PRODUCTS.** As seen in the latter diagram, once pyruvic acid is formed, it may undergo 1 or more of several transformations, assimilative or dissimilative, depending on the species of cell and the physical and chemical conditions of the environment. While it is not important, at this point, to understand the chemistry indicated in the diagram or to memorize the scheme, it is necessary for the microbiologist to know something of the different end-products of energy metabolism in different species of cells. A good illustration is the common, saprophytic, gram-negative rod, *Klebsiella aerogenes*.

In the case of this organism the following final products of glucose dissimilation under ordinary cultural conditions have been demonstrated to occur: hydrogen, carbon dioxide, ethyl alcohol, acetic acid, acetyl-methyl-carbinol, 2-3-butylene glycol, trimethylene glycol, lactic acid, glycerol and succinic acid. Whether all of the reactions that occur in their formation are exactly as indicated in the diagram is not certain, but many are well established. Some of the end-products of metabolism, for example acetyl-methyl-carbinol,  $\text{H}_2$  and  $\text{CO}_2$ , are easily tested for in a culture tube and are often very distinctive of certain species and, therefore, are of use in identifying certain microorganisms. We have already indicated that some of these products are of great industrial value and will be referred to later in the chapter on Industrial Microbiology.

**ALTERNATIVE METABOLIC PATHWAYS.** An important concept in this connection is that of *alternative metabolic pathways*. Although a series of physiological chemical reactions, such as those diagrammed to occur in the utilization of glucose, may usually follow a definite sequence, this is not necessarily fixed. Under altered conditions of nutrition or environmental factors, (e.g., pH, temperature, presence of essential metabolites or substitutes, "blocks" such as antibiotics, sulfonamides, etc.), a different set of reactions may occur by different chemical pathways, and with different growth rates, waste products, etc. The alternative pathways may be more or less effective in the release of energy and synthesis of cell substance than the normal pathways.

**Facultative Respiration.** Most of the common types of bacteria and yeasts

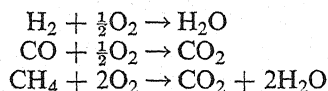
are capable of utilizing both atmospheric oxygen and reducible compounds as final hydrogen acceptors. They possess both aerobic and anaerobic respiratory mechanisms. They are said to be *facultative* with respect to respiration since they have the faculty of growth under either condition.

**Direct Oxidation.** Some species of microorganisms apparently can bring about a direct combination between atmospheric oxygen and the substrate molecule. An example sometimes given is the oxidation of the alcohol in hard cider, wine, beer, etc., to acetic acid as in vinegar manufacture. The bacteria used in this industrial process are *Acetobacter* species (see Chapter 44). The over-all reaction is:



In effect, this is in part a dehydrogenation followed by oxidation of the hydrogen. But oxygen is also added to the substrate.

There are several species of microorganisms which obtain energy by the oxidation of such substrates as molecular hydrogen, carbon monoxide, sulfur, methane, ammonia, etc. For example,



In this sort of respiration free, atmospheric oxygen appears to oxidize the substrate directly (via enzyme action). Dehydrogenation does not appear to be involved.

All of the biological oxidations described above proceed relatively slowly, in a stepwise fashion, molecule-by-molecule, so that no explosively rapid and damaging release of energy occurs as might be expected, for example, when hydrogen is made to combine with oxygen to form water (see reaction given above). All proceeds quietly and gently but efficiently because the reactions are regulated by enzymes.

**Oxidation-Reduction.** When we discuss anaerobes later on (Chapter 34) it will be worth remembering that when any substrate molecule is oxidized, some other substance is reduced. Every biological oxidation is accompanied by a simultaneous reduction, just as every action is accompanied by a reaction.

## 2. NUTRIENTS AS SOURCES OF CELL BUILDING MATERIALS

It is evident that the various types of organisms differ greatly in their nutrient requirements. Some autotrophic organisms, like *Nitrobacter* in the soil, have very great synthetic ability, forming amino acids, proteins, fats, carbohydrates, enzymes, vitamins, and all of the many other complex organic compounds which make up a living cell, from  $\text{CO}_2$  of the atmosphere and a solution of inorganic compounds similar in composition to sea water. Some common heterotrophic organisms possess almost as great a synthetic ability. They differ from the complete autotroph described above only in requiring, in addition to the simple, mineral solution, one organic food substance such as glucose or sodium citrate, as a source of carbon and energy.

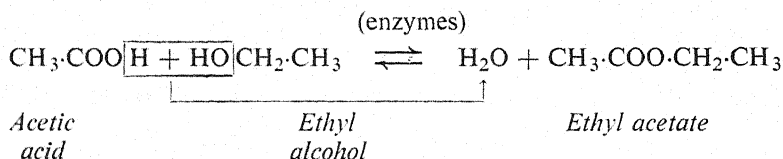
In contrast to these "almost-autotrophs," many heterotrophs are highly restricted and demanding in the matter of foods. Such are represented by human beings or, among microorganisms, by various species of parasitic

bacteria like gonococci or influenza bacilli. These *must* have blood or tissues, or complex organic extractives thereof, which they cannot synthesize for themselves because of their enzymic limitations.

**Assimilation.** In the synthesis of cell substance certain atoms and molecules from the food substances are selected, rearranged, and put together again in new structures constituting the protoplasm, cell wall, and other portions of the new organism. This is *assimilation*. Unused residues of the nutrient molecules are given off as wastes.

The synthetic reactions involved are *endothermic* since energy is stored in the complex molecules resulting from the building processes. The energy is derived from the exothermic dissimilative or respiratory reactions previously described.

Some of the endothermic reactions, viewed in an over-all sense, appear to be the reverse of hydrolysis, i.e., complex molecules seem to be built up from pairs of simpler ones by the abstraction of hydrogen from one and oxygen from the other of the pair. A molecule of water is formed from each pair of complex molecules brought together. An example is given below.



However, in general, biosynthetic processes appear to be much more complex and to involve a number of chemical steps other than the mere reverse of hydrolysis. The entire synthetic process is not yet completely known.

With respect to the energy involved, an analogy is seen between assimilation and the building up of a column of bricks. Each brick piled up is analogous to a chemical synthetic step in the building up of some energy-bearing substance in the cell, such as starch or fat. Potential energy is accumulated in the column of bricks and in the molecules of starch or fat. When the column of bricks is knocked down energy is liberated in the form of heat, sound, motion, perhaps light, etc. When the fat or starch is broken down chemically (oxidized or dissimilated) by the cell, the energy released is manifested as life.

**DUAL-PURPOSE UTILIZATION OF NUTRIENTS.** It has been found that organic nutrients like glucose and amino acids are generally not used exclusively for either carbon or energy. Parts of the nutrient molecules may be incorporated into the cell substance "as is," while other parts are oxidized as sources of energy and given off as waste. The total caloric energy in a molecule of nutrient such as glucose is, therefore, not necessarily an exact indication of how much energy it will yield to a given type of cell.

### 3. NUTRIENTS AS ACCESSORY SUBSTANCES\*

As indicated in a foregoing paragraph these are substances which do not yield energy to the cell or contribute materially to its bulk, but which are effective in very minute quantities and absolutely essential to growth. They range in structure from simple elements to complex organic compounds.

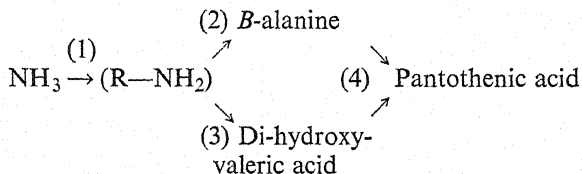
\* These are also often spoken of as *essential metabolites*, *nutrilites* or *growth factors*.

Vitamins are good examples of growth factors, as well as certain metallic elements.

Certain amino acids also have the status of growth factors; that is, they are among the substances absolutely essential to the growth of a number of bacteria. A good example is tryptophan, without which no growth of the typhoid bacillus (*Salmonella typhi*), the lockjaw organism (*Clostridium tetani*), the diphtheria bacillus (*Corynebacterium diphtheriae*) and several others can occur, even though their culture medium be complete in all other respects. On the other hand, there are many bacteria which can synthesize their own tryptophan and so do not need to have it fed to them. Among these are autotrophic bacteria and numerous others the cells of which, though grown in a tryptophan-free medium, nevertheless contain this amino acid.

A number of other growth factors are known or are under investigation. Among them are inositol, hemin, glutamine and vitamins such as nicotinic acid, riboflavin, thiamine, and pantothenic acid.

One probable role of such compounds in bacterial nutrition was described by Fildes as early as 1939. Assume that an organism requires pantothenic acid, a vitamin. It is able to carry out all of the preliminary steps necessary to the formation of pantothenic acid, beginning with simple ammonia (see steps 1-3 in the diagram below). However, it is not able to synthesize beta alanine (one of the amino acid radicles in the complex pantothenic molecule). This is indicated in step 2 in the diagram. Given a minimum quantity of *B*-alanine, completion of the pantothenic acid is provided for (step 4) and growth occurs. Without *B*-alanine, no growth is possible.



A strain or species able to synthesize *B*-alanine as well as the other components of pantothenic acid encounters no such difficulty and we say that "it does not require *B*-alanine." Both may require *B*-alanine, but one can manufacture it internally, the other cannot.

#### CULTURE MEDIA

**Natural and Empirical\* Culture Media.** In the early days of microbiology natural culture media were widely used: milk, urine, diluted blood, vegetable juices, etc. Some media were artificially prepared: beef or vegetable broths, meat "infusions," and the like. Such media are still widely used. They contain a rich assortment of soluble organic and inorganic compounds comprising all of the elements and accessory substances for many (but not all) microorganisms. Such media are convenient, inexpensive, and serve for many routine purposes.

**Synthetic Culture Media.** Some culture media, developed as a result of increased knowledge of microorganismal nutrient requirements, consist

\* Used on the basis of experience and not on the basis of exact knowledge of their composition and action.

wholly of dilute, accurately known and reproducible solutions of chemically pure, inorganic and/or organic compounds. They have special uses in research, industry and the like. Artificial media of exactly known, reproducible composition are called *synthetic media*. In order to use them it is necessary to know the exact nutritional requirements of the organism for which they are designed. Such knowledge is, unfortunately, available for relatively few organisms.

**Living Culture Media.** Living culture media consist of live, animal or vegetable cells cultivated in special nutrient fluids. The live cells serve as foods especially for viruses and rickettsiae which, as previously noted, cannot multiply in inanimate, artificial culture media.

Examples and uses of each of the foregoing types of culture media are given in the following discussion of cultural methods.

#### CULTURAL METHODS

It is evident from the foregoing that no single cultural medium or method is suitable for all forms of microorganisms. Space would not permit a presentation of the thousands of kinds of culture medium which have been devised for the many various objectives of microbiology. For illustrative purposes we will describe some of the media most commonly used for microorganisms described in this book. These are representative of many others, which are mere modifications of the same general types.

1. **Synthetic Inorganic Media.** These are among the simplest of media. A medium used for a common, sulfur-oxidizing autotrophic species of bacteria found in the soil (*Thiobacillus thiooxidans*) is as follows:

$(\text{NH}_4)_2\text{SO}_4$ .....	0.2 gm
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.5 gm
$\text{KH}_2\text{PO}_4$ .....	3.0 gm
$\text{CaCl}_2$ .....	0.25 gm
Powdered sulfur.....	10.0 gm
Distilled $\text{H}_2\text{O}$ .....	1000 gm

The phosphate in the medium is a buffer and maintains a suitable pH. It also serves as a source of phosphorus and potassium for use in the cell. Additional trace elements (Cu, Fe, Mn, Na, and others) are usually present as impurities in the water or ingredients. The powdered sulfur is the source of energy. Incubation is at about 25° C, common in the soil in summer. Note that there is no source of carbon and that nitrogen is provided in an inorganic form. Carbon is obtained as  $\text{CO}_2$  in the atmosphere. This is a typical, synthetic, inorganic medium. That is, each of the ingredients is a chemically pure, inorganic substance, the exact chemical composition of which is known and under the control of the worker. Such media (as well as any other fluid media) may be solidified with agar\* or silica gel, the choice depending on the organisms. Some autotrophs are inhibited by organic substances such as agar.

\* A carbohydrate having some of the properties of gelatin in that, dissolved in water in about 2 per cent concentration, it fluidifies on boiling and sets to a firm jelly at about 40° C. It is attacked by few common bacteria and is therefore useful for solidifying bacteriological media. Silica gel is often used in its place to solidify media for strict autotrophs since it is inorganic. Strict autotrophs cannot grow in the presence of organic matter.



2. **Synthetic, Organic Media.** By way of contrast, a synthetic medium devised for a rather fastidious, pathogenic heterotroph, *Corynebacterium diphtheriae* (cause of diphtheria), is given as follows:

SYNTHETIC MEDIUM FOR *C. DIPHTHERIAE* (Pappenheimer, Mueller and Cohen).

*Solution A*

glycine.....	.5 gm
valine.....	1.0 gm
leucine.....	.5 gm
glutamic acid.....	5.0 gm
methionine.....	.2 gm
tyrosine.....	.1 gm
NaCl.....	5.0 gm
K <sub>2</sub> HPO <sub>4</sub> .....	2.0 gm
Water.....	500.0 ml

*Solution B* (add to A)

cystine (an amino acid) . . . . .0.2 gm (in 30 per cent HCl, minimal quantity to dissolve).

*Addition 1* (add to A after adding B)

Vitamins {	pimelic acid.....	1 mg
	beta alanine.....	1 mg
	nicotinic acid.....	2 mg

Adjust to pH 7.8. (This is done by adding dilute NaOH to the desired pH.)

*Solution C* (add 0.3 ml of this to A, after B and 1 and pH adjustment)

Source of Ca <sup>++</sup> {	CaCl <sub>2</sub> .....	33 gm
	H <sub>2</sub> O.....	100 ml

Boil gently 10 minutes. Filter through paper.

*Addition 2* (add to the above)

Sources of Mg <sup>++</sup> , Cu <sup>++</sup> , S. {	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	.3 gm
	CuSO <sub>4</sub> ·5H <sub>2</sub> O.....	5.0 mg
	tryptophan (an amino acid).....	100 mg
	water to make total volume of 1000 ml.	

Dispense in flasks and autoclave.\*

Just before inoculation, to each 100 ml add aseptically 2 ml of

*Solution D*

Organic sources of	sodium lactate (Merck U.S.P.).....	37.0 ml
energy and carbon {	glucose C.P.....	7.5 gm
	maltose (purified).....	15.0 gm
	CaCl <sub>2</sub> .....	0.3 gm
	H <sub>2</sub> O to make.....	100.0 ml

Autoclave.

The advantages of synthetic media are that they are exactly reproducible and, in some instances, much less expensive and troublesome to prepare than media made with meat and meat products such as peptone. Also, they do not contain proteins and therefore have no antigenic or allergenic properties when injected into man or animals in vaccines or for experimental purposes. By virtue of these properties they lend themselves well to exact experimental research and to medical and commercial uses. (See sections on vitamin assay, Chapter 44.) The great difficulty is that a solution suitable for one species is usually not suitable for another, and it is often difficult to determine the exact requirements for a given species.

\* Sterilize with compressed steam.

**Empirical, Complex, Organic Media.** While synthetic media have much to recommend them for special work they are not so commonly used for routine purposes as less exact media because (1) they are often expensive and time consuming to prepare and (2) we know the exact requirements of relatively few microorganisms. It is, therefore, customary in most laboratories to use less exact organic media which, as we know from experience, support good growth of a wide variety of organisms (except strict autotrophs inhibited by organic matter). Such organic mixtures are very useful, not too expensive and quickly prepared. Many are commercially available in dehydrated ("add water, heat and serve") form from laboratory supply houses.

**PEPTONE.** Such media nearly always contain meat- or casein-digest derivatives such as *peptone*. These have in them all the basic mineral content of living material as well as organic carbon and organic nitrogen in numerous complex and soluble forms. Such media furnish a wide variety of substances derived from living material and satisfy a wide range of nutritional requirements. Examples of two commonly used media of this type are given here.

**MEAT EXTRACTS AND INFUSIONS.** "Beef tea" (beef extract) or an aqueous extract made by soaking (*infusing*) ground meat in water are common sources of culture media for a wide variety of microorganisms. They are rich in minerals, vitamins, proteins, carbohydrates, etc. They are often mixed with peptone, a buffer and sterilized after adjusting the pH to near neutral. Media made with "beef tea" are called *extract* media; those made from fresh meat are called *infusion* media. Both, or any or all, media are sometimes loosely included in the general term "nutrient broth" or "nutrient solution."

Meat is not the only useful source of organic matter. Extracts of vegetables of various kinds are often used. Media made with vegetable extracts are available ready-prepared in convenient tablet form. Some workers use the flesh or juices of shell fish, while those interested in the bacteria of milk sometimes use whey, skim milk, etc. Eggs are often used also, especially for tubercle and diphtheria bacilli. An infusion of partly digested soybeans has been shown to be as good as meat infusion for many purposes. Some media are made by adding bits of kidney, spleen, or other tissues freshly removed from dead animals under aseptic precautions to tubes of broth.

**ADSORPTION OF NUTRIENTS AT SURFACES.** The incorporation, in bacteriological culture media, of small amounts of some solid substance such as ground meat, or even sand, is often advantageous as many microorganisms seem to grow best in the crevices of, or in contact with, the surfaces of such matter, forming little nests or *niduses* there. It is probable that oxygen and food substances concentrate at such points by adsorption. Furthermore, digestive enzymes secreted by the microorganisms into their environment to digest foodstuffs do not diffuse away so quickly (Fig. 13-3) in such protected *niduses*. Indeed, in very dilute media, growth may occur only at such surfaces.

**Special Media.** To any of the organic media various test and experimental or nutrient substances may be added. Certain carbohydrates may be included to test the fermentative powers of various organisms. Organic esters, blood, glucosides, and many other compounds are put into the medium for a great variety of experimental purposes. The medium is then referred to by the name of the special substance; for instance, "blood-infusion-broth"; "serum-dextrose-extract-agar"; "starch-carrot-whey-agar" and so on.

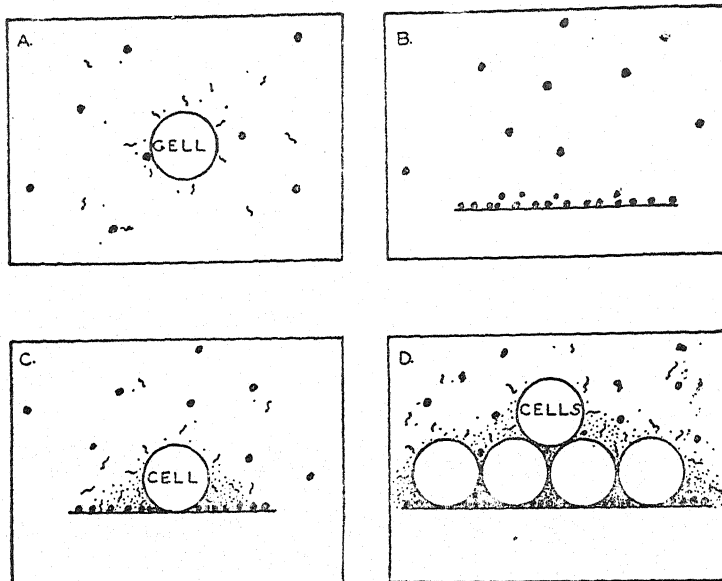


Fig. 13-3. *A*, a free-floating bacterial cell surrounded by a few suspended particles of food (dark circles) which must be hydrolyzed by the exoenzyme (helicoidal lines) before the resulting hydrolysates (dots) can be assimilated. *B*, particles of food concentrated in a monomolecular layer on a solid surface. *C*, food particles are more available to the cell on the solid surface where the interstices at the tangent of the bacterial cell and the solid surface retard the diffusion of exoenzymes and hydrolysates away from the cell. *D*, multiple cells form additional interstitial spaces. (ZoBell, Claude E., in *J. Bact.*, vol. 46.)

#### PURE CULTURE METHODS

**Difficulties with Mixed Cultures.** Early microbiologists had to contend with the difficulty that as soon as anyone attempted to cultivate organisms from a particular source such as blood, soil, a lesion on a plant, or to handle them with instruments, the microorganisms under study became mixed with a variety of extraneous microorganisms from the instruments, from dust, or from some other source. It was often impossible to distinguish one kind of microorganism from another in such contaminated cultures, since many entirely different kinds look exactly alike when viewed with the microscope, even when stained by Gram's method. Pasteur, Koch and many others had the same difficulty. No one could be sure that the chemical or physiological properties he assigned to a given culture were due to one kind of microorganism alone and would remain constant or whether the observed reactions were due to a combination of organisms growing together in a culture vessel. It was like trying to determine the properties of a salt or element in solutions which contained other salts and elements. Such mixed cultures would change their properties as soon as one or the other of the different kinds of microorganisms died off or gained the ascendancy. But no good method of separating or *isolation* of organisms was then known.

**Origin of Pure Culture Technique.** Koch had observed the growth of molds and of different sorts of bacteria in isolated masses (called *colonies*) of various colors on slices of decaying potato in his wife's kitchen. One day, being in an

investigative mood, he thrust a sterile platinum wire into one of the gray bacterial masses and put a bit of it in a little water under his microscope. He saw that all of the bacteria in this particular colony looked exactly alike. Koch examined the bacteria from a yellow colony on the potato, and then those of a red one and of a violet one. The organisms from one colony were all round; from another they all had the appearance of tiny, immobile cylinders; from a third they looked like minute, living, highly motile, spiral springs; but all the microorganisms in any one colony were always exactly the same. It was obvious that, by cultivating microorganisms on solid food, he could obtain isolated colonies of any single kind; a *pure culture*!

**The Use of Gelatin.** Extending this principle it was but a step to the use of gelatin to prepare a transparent, solid, sticky, nutrient surface on flat pieces of glass. In addition, various nutrient infusions and test substances could be added to the gelatin before it was allowed to "set." Here was a very important advance, a revolutionary advance, one that has been the basis of all our present-day bacteriology. Thenceforth the study and discovery of bacteria in *pure culture* and by relatively exact methods became a matter largely of patience and hard work.

In summer, however, and when held in body-temperature incubators, the gelatin melted and this spoiled everything. Being a protein it was often digested and fluidified by the metabolic processes of the bacteria. Besides that, particles of dust settled on it with various microorganisms from the air or soil which contaminated it, obscuring and confusing the results as badly as ever.

**First Use of Agar-Agar.** Many students flocked to Koch's laboratory from all over the world to learn his methods. One of these was W. Hesse. To the wife of this man the science of microbiology is indebted for suggesting, in 1883, as a substitute for gelatin, the jelly-like substance agar-agar, derived from seaweed and used at that time for making jellies. Agar is transparent, colorless, is not digested or liquefied by most bacteria, melts only at boiling temperature and once melted, does not set again till about body temperature. Agar has not been improved upon as a solidifying agent for culture media and is in general use for this purpose today.

**Origin of the Petri Plate.** In order to prevent contamination of the pure cultures by dust, another student in Koch's laboratory, R. J. Petri, suggested the simple expedient of pouring the melted, nutrient agar into circular, shallow dishes and immediately covering them with a glass cover. This permitted prolonged examination of the cultures but excluded dust. Such dishes are widely used today and are called Petri plates.

The preparation and study of pure cultures then proceeded at a great rate all over the world.

For many years the use of Petri dishes imposed a burden of cost and dish-washing on bacteriological laboratories. Plastic, disposable dishes of this type are now available and, for many purposes, solve these knotty and long-standing problems.

**Preparation of Solid Media.** The preparation of solid media is comparatively simple. To any of the fluid organic media prepared as indicated above, gelatin may be added in 10 per cent concentration while the fluid is still hot. Since, as Koch found, this liquefies so readily, agar is usually substituted

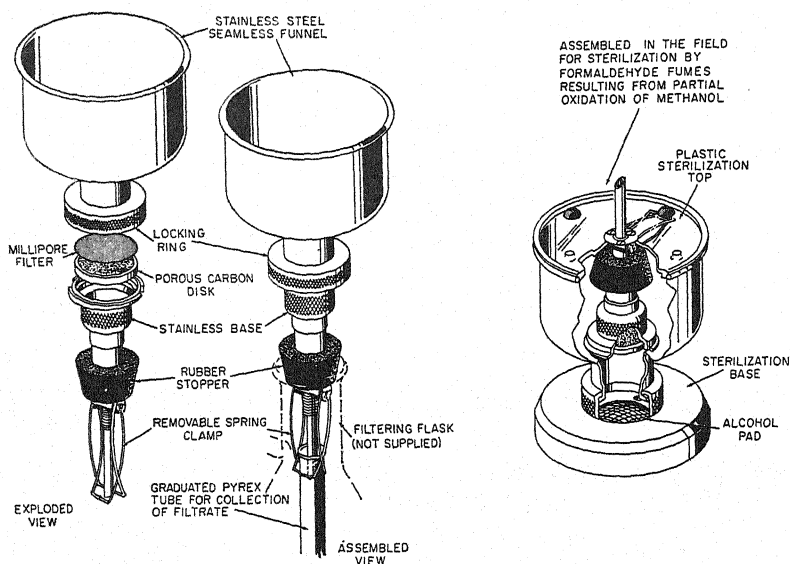


Fig. 13-4. One form of filtering device for mounting a Millipore filter disk. The porous carbon base is placed beneath the fragile filter disk for support. It plays no role in the filtration process. In the field assembly, sterilization by formaldehyde fumes is suggested since the membranes are sensitive to heat except under very carefully controlled conditions in the laboratory. (Courtesy of the Millipore Filter Corporation, Watertown, Mass.)

following Frau Hesse's suggestion. Agar is used in 1.3 to 2 per cent concentration.

**Silica Gels.** Silica can be made into jelly-like material with surfaces sufficiently solid to be inoculated like agar or gelatin. The advantages of silica as a solidifying agent are that, supplemented with an appropriate mixture of inorganic nutrient minerals, it will support the growth of colonies of a number of species of autotrophic soil microorganisms which are injured by organic substances, such as the agar, eggs, serum or gelatin ordinarily used as solidifying agents. Silica gels are much used by microbiologists studying bacteria of the sea or soil.

**Cultivation on Fine Pore Filters.** A means of cultivating bacteria on a solid surface without the use of a special solidifying agent has been developed through the use of very thin, porous membranes of cellulose acetate, collodion or similar materials. Such membranes\* can be prepared with pores of the magnitude of  $0.5 \mu$  or smaller. Mounted on a perforated plate to support it in a funnel-shaped apparatus such a membrane acts as a sieve to filter bacteria, or even viruses, from any fluid as desired: drinking water, dilute feces, blood, digested, centrifuged sputum, dilute broth cultures, etc. (Fig. 13-4). After the fluid has passed through, depositing the microorganisms on the surface of the membranes (Fig. 13-5), the membrane is carefully removed with sterile forceps from the supporting device and laid on a disk or pad of sterile blotting paper saturated with any desired nutrient solution. The nutrients diffuse

\* Available commercially.

through the membrane and support growth of colonies on its surface just as though these were on an agar surface. Various selective or special media may be used to cultivate special sorts of bacteria. The disks of cellulose acetate or collodion are sometimes sterilized by exposure to ethylene oxide or carboxide, but may also be sterilized by heat with suitable precautions recommended by the manufacturers.

Interesting deviations from the standard procedures of cultivating bacteria are possible. One may incubate organisms on a membrane laid on a pad moistened with one solution for a time, and then transfer the membrane, with its colonies, to a different medium, or to a stain or to a pad saturated with a reagent to test for some particular growth product or property of the organisms growing on the membrane. One may easily filter a liter of river water through a membrane, place the membrane on a pad containing a special selective medium, or a general-purpose nutrient broth, and isolate typhoid bacilli from the water or enumerate the cultivable organisms in the total sample. Many profitable applications and intriguing possibilities will suggest themselves to the industrious and ingenious student, once he starts working with the filters. (See also Chapt. 37.)

**Selective Cultivation.** This was discussed in Chapters 3 and 4 in connection with yeasts and molds. The basic principle is simple: preparation of a medium which will support good growth of the desired organism but containing substances to suppress the growth of undesired organisms. For example, in isolating gram-negative *Salmonella* (typhoid) and *Shigella* (dysentery) bacilli from feces of patients (Chapt. 39) a selective "indicator" dye, 2, 3, 5 -triphenyl tetrazolium chloride, is added to nutrient medium. This permits growth of many gram-negative rods but inhibits many gram-positive species common in feces. In isolating the gram-negative *Brucella* (undulant fever) organisms from heavily contaminated soil or manure suspensions a good nutrient agar is used

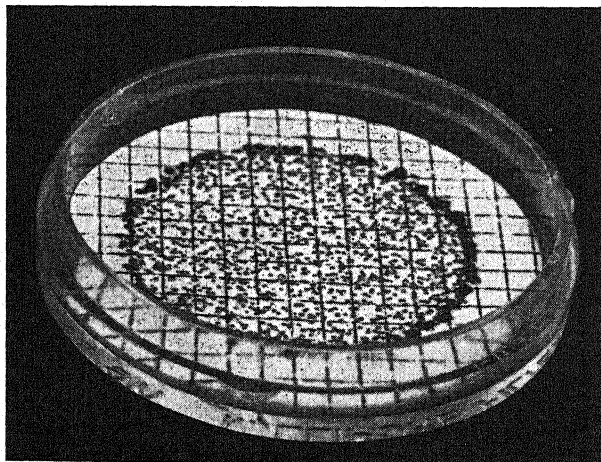


Fig. 13-5. Disposable plastic Petri dish containing a used Millipore filter disk resting on a pad saturated with culture medium. The whole has been incubated and the bacteria caught on the disk have grown into visible colonies. Actual size. (Courtesy of The Millipore Filter Corporation, Watertown, Mass.)

to which are added: Polymyxin B, Actidione, Bacitracin, Circulin and crystal violet, with good effect. The first four are antibiotics, the last a dye, none of which inhibits *Brucella*. Scores of similar selective media are to be found in the literature.

**Enrichment.** In this process inhibiting agents are not ordinarily used. Conditions are made particularly favorable for one particular species in a mixture. Continued incubation results in predominance ("enrichment") of the desired species.

#### USE OF LIVING CELLS FOR CULTIVATION OF MICROORGANISMS

Virtually all yeasts and molds, most PPLO, some protozoa and, with the exception of some Spirochaetales, Chlamydobacteriales and sulfur bacteria, all bacteria are known to be cultivable upon lifeless artificial media; i.e., upon material *devoid of living cells*.<sup>\*</sup> Some of these microorganisms, mainly pathogens, require dead tissue or inert body fluids such as blood serum or ascitic fluid for optimum growth. While many will also grow in contact with living tissue and in so doing sometimes cause disease, none is *restricted* to live tissue. This is one of the properties which distinguish viruses and rickettsiae from all of the above-mentioned groups of microorganisms. Viruses and rickettsiae are *obligate parasites*; i.e., they cannot multiply outside of, or in the absence of intimate contact with, living cells.

#### PROPAGATION AND CULTIVATION OF VIRUSES AND RICKETTSIAE†

Viruses and rickettsiae are often propagated in their natural hosts; that is, by Nature's method of infection. In the laboratory we imitate Nature by transmitting infectious material (blood, serum, pus, tissue, sap of plants, etc.) from one animal or plant to another by injections, scratches, punctures insect bites, etc. This is frequently necessary for research but it is laborious, expensive and sometimes very dangerous to laboratory personnel unless they can be vaccinated against the organism they are working with, as, for example, Rocky Mountain spotted fever rickettsiae or yellow fever virus. This sort of propagation, in live creatures, is usually called *propagation in vivo*.

**Tissue Cultures.** *In vitro* propagation (in culture flasks, tubes, etc.) eliminates the use of living animals (or plants). It does not eliminate the necessity for living animal or plant *cells*. *In vitro* propagation of viruses and rickettsiae thus requires knowledge of how to cultivate living cells *in vitro*. A culture of tissue cells *in vitro* is called a *tissue culture*. Methods of cultivating animal tissues will illustrate the basic ideas. For plant tissues appropriate modifications are made in details such as nutrients supplied, temperature, etc. Bacterial viruses (bacteriophage) require special, modified procedures which have been discussed in Chapter 6. All tissue culture work requires the most strict precautions and considerable technical skill to keep out contaminating bacteria, molds, yeasts, etc. These can grow vigorously in the nutrient solutions furnished for the tissue cells. This, of course, is detrimental. Antibiotics are generally used to suppress them.

<sup>\*</sup> Erythrocytes may be regarded as lifeless since they carry on no known metabolic processes in bacteriological media.

<sup>†</sup> Methods for cultivating rickettsiae are included at this point since they are the same, basically, as those for cultivating viruses.

The viruses grow in the multiplying tissue cells. As the infected cells disintegrate the viruses and rickettsiae are liberated into the suspending fluid. The tissue cells show visible changes and, seen daily under the microscope, the progress of their infection and disintegration can readily be followed. Viruses which thus damage tissue cells are said to be *cytopathogenic* (Fig. 13-6).

**PLASMA CULTURES.** Tissue cells may readily be cultivated in plasma\* clots. Briefly, a drop of sterile, fluid plasma is mixed with a nutrient fluid containing serum† (20%); extract of embryonic tissue (5%); Earle's solution (70%). A tiny fragment of the freshly cut tissue to be cultivated is then placed in the center of the plasma drop on a glass slide. Clotting occurs almost immediately. The drop is then placed in a tightly-sealed, glass container and held at 37° C. Transfers of the growing tissue to new plasma drops (*explants*) must be made every two or three days. Growth of the tissue is observed on the slide with the microscope.

Such cultures have limitations due to their minute size. Excellent slow-motion pictures have been made of polio virus cultures in plasma clots, showing effects of the virus on the cells.

**ROLLER TUBES.** In a modification of the plasma culture called *roller-tube cultures*, tubes or bottles of any desired shape and size are wetted inside with plasma. A score or more of fragments of live tissue are then placed in the plasma. When clotting has occurred nutrient fluid is placed in the vessel which is then sealed. It is placed in a nearly horizontal position and rotated in a "roller" at the rate of about 7 r.p.m. in an incubator at 37° C (Fig. 13-7). The tissues are thus bathed in the nutrient fluid seven times per minute as the bottles rotate. These cultures can be maintained for long periods by replacing the nutrient fluid every two or three days. Relatively large amounts of tissue and of virus may thus be obtained and prolonged experiments are possible.

In 1950 Robbins, Enders and Weller cultivated the virus of poliomyelitis in monkey-kidney tissue by such methods and thus made the basic observation from which the Salk polio vaccine was derived. Viruses of herpes simplex (fever blister), feline pneumonitis (a large virus), influenza, and numerous others have been cultivated *in vitro* by similar methods.

It is of interest to note that cancer (neoplasm‡) cells can be cultivated in tissue cultures and that several viruses are highly fatal to such cells. Many persons have thought of using viruses *especially adapted to neoplasm cells* (but *harmless* to normal cells) to cure neoplasms in human beings. Very exciting results have been obtained. These hold promise, but the method requires much more study.

**CULTURES ON GLASS.** Clot-culture methods were long used because mammalian cells grow best in contact with some solid support. It was later found that they grow well directly on glass, spreading in a sheet over the inner surface of flasks or tubes. This method, in numerous variations, is now widely used. In a typical procedure, *susceptible* tissues are minced and suspended in a

\* The fluid portion of the blood after removal of all white and red cells. Plasma can clot, just as does whole blood.

† The fluid portion of the plasma after clotting (fibrin formation) has occurred.

‡ *Neo* is from a Greek root meaning new; *plasm* is from a Greek word meaning formations. A neoplasm is a new growth or new tissue.



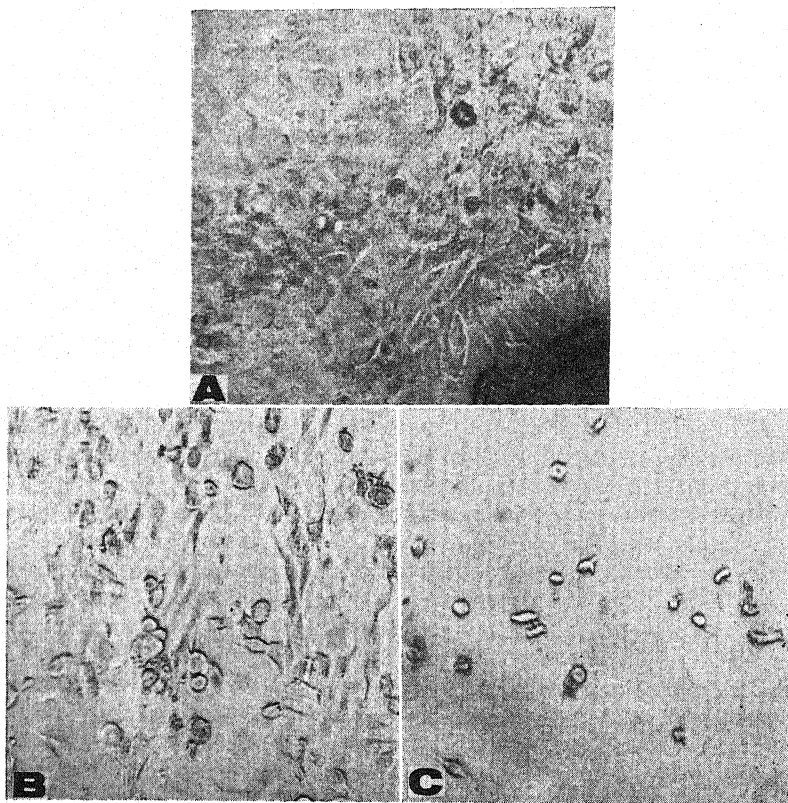


Fig. 13-6. Cytopathogenic effect of poliovirus (type I) on monkey-kidney tissue cells in a roller-tube culture. In *A* are seen normal, young cells forming a continuous, cellular structure. The cells are stellate in form and the nuclei visible in most, but not conspicuous. In *B* the same preparation is seen 48 hours after infection with poliovirus. Note open spaces where cells have separated and/or been destroyed, "rounding" of the cells and conspicuous, denser nuclei. In *C* the same preparation is seen 4 days after the infection. Almost total destruction of the tissue cells has occurred. The virus has multiplied enormously. (Photos courtesy of Dr. Morris Schaeffer, Virus Laboratories, Communicable Disease Center, U. S. Public Health Service, Montgomery, Alabama.)

growth medium commonly consisting of Earle's or Hanks' solution\* at pH about 7.2 (75 per cent); about 20 per cent *suitable* mammalian serum; some type of extract of *selected* embryonic tissue (5 per cent); antibiotics. Partly synthetic media are being used more as knowledge of tissue-cell requirements accumulates. These must contain *at least*: 7 specified vitamins; 6 salts including buffers; 13 specified amino acids; serum; glucose. (See references.)

Trypsin is added to the suspension of minced tissue to digest non-living material and separate the cells. Action of the trypsin is stopped in fifteen

\* Earle's solution (Compare with media used for autotrophic bacteria and mineral content of human blood!):

NaCl.....	6.8 gm	NaH <sub>2</sub> PO <sub>4</sub> .....	0.125 gm
KCl.....	0.4 gm	NaHCO <sub>3</sub> .....	2.2 gm
CaCl <sub>2</sub> .....	0.2 gm	Glucose.....	1.0 gm
MgSO <sub>4</sub> .....	0.2 gm	H <sub>2</sub> O to.....	1000 ml

minutes by soy-bean trypsin inhibitor. The tissue cells are then removed from the fluid by centrifugation. The "clean" cells are now resuspended in fresh culture medium and placed in small tubes held motionless in a slanting position at 37° C overnight. In twenty-four hours the cells attach themselves to the glass and start to grow. The culture may then be handled as are roller tubes previously described.

#### CULTIVATION IN CHICK EMBRYOS

Fertile eggs must be used. They are incubated as for hatching until the embryo is well developed (from five to fourteen days). The *shell* is cut by means of a dentist's fine carborundum disk on a flexible shaft, rotated by a dental motor (Fig. 13-8). The square of cut shell is then gently lifted off with a sterile forceps, exposing the shell membrane immediately underneath. The shell *membrane* is pierced, the chorioallantois drops away from it. The shell membrane may now be torn off like paper against the cut edge of the shell, leaving the vascular embryonic membranes exposed for inoculation.

This is done simply by applying the inoculum with a loop, a dropper, or other gentle means. The hole in the shell is surrounded by sterile melted petrolatum or paraffine and immediately covered with a sterile coverslip of glass, cellophane or gummed transparent plastic strip. The egg may then be incubated as for hatching.

Modifications of this method of inoculation include injection through the membrane directly into the amniotic and chorionic cavities, yolk sac, em-

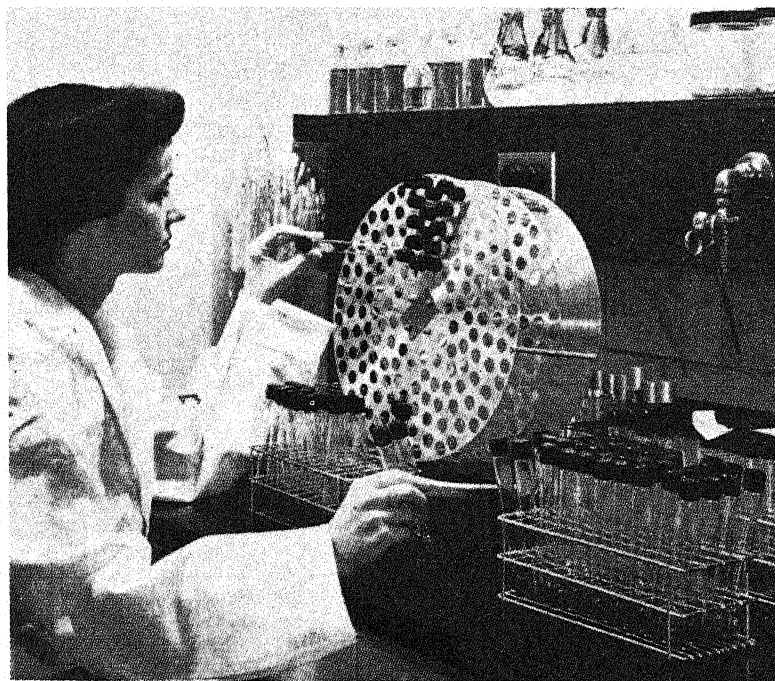


Fig. 13-7. Roller-tube technique of tissue culture. Poliomyelitis virus used in the preparation of vaccine is being tested in tissue cultures, which the virologist is placing in the roller-tube apparatus prior to incubation. (Parke, Davis & Co.)

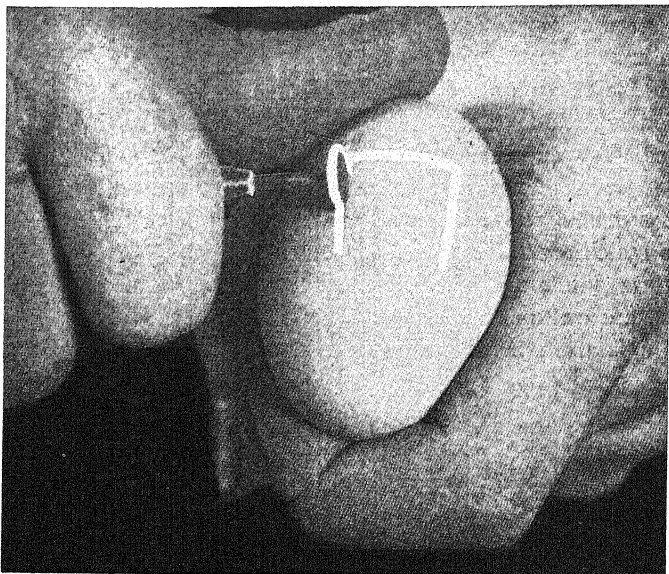


Fig. 13-8. Cutting window in shell with rotating carborundum disk. (Goodpasture and Buddingh, *Am. J. of Hyg.*, vol. 21.)

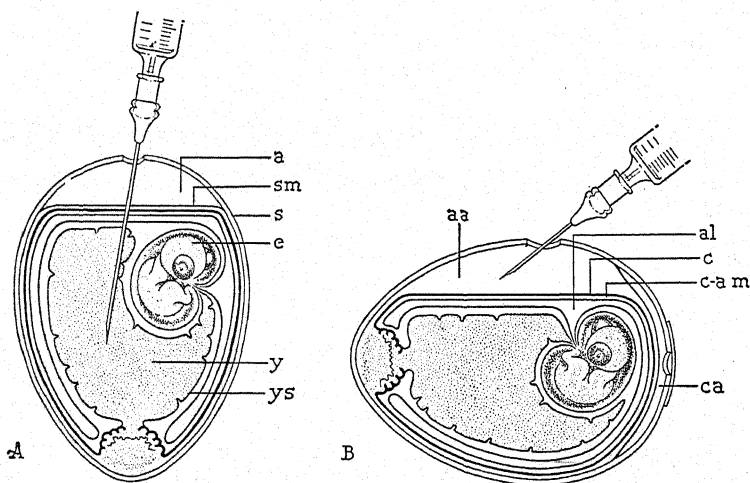


Fig. 13-9. Methods of inoculating chick embryos. *A*, injection into the yolk sac for cultivation of rickettsiae. The normal air space is seen at *a*, the shell membrane at *sm*, and the shell at *s*. The living chick embryo is shown at *e*, while the yolk sac and yolk are seen at *ys* and *y*, respectively. *B*, many microorganisms can infect the chorioallantoic membrane, seen at *c-am*. An artificial air space (*aa*) is made by allowing air from the normal air space to escape through the small opening seen at the right end of the egg. The collapsed air sac (*ca*) is then sealed up. Other important parts of the embryonic membranes and sacs are the allantoic sac (*al*), and the chorion (*c*). (Courtesy of E. R. Squibb and Sons. From Kelley and Hite.)

bryonic tissue, etc. For some of these purposes a single tiny hole in the shell is all that is necessary (Fig. 13-9). Many saprophytic microorganisms, especially yeasts, molds and bacteria, which may enter as contaminants are able to multiply as well in the fluids of the chick embryo as in the culture tube. The embryo is usually killed and destroyed by such contaminants. Extraneous contamination with such organisms from the air, shell dust, implements, or from contaminated inocula such as feces or saliva, is therefore an ever present source of error in the use of chick embryos for the study of viruses and rickettsiae. As in handling tissue cultures, rigid precautions against such contaminations and considerable technical skill are required. It is customary in both tissue cultures and egg cultures to mix small amounts of penicillin and streptomycin, or other antimicrobial drugs with such materials before injection. These agents control bacterial contaminants but permit the growth of viruses and rickettsiae since these are not affected by the drugs.

## REFERENCES

- Atkinson, D. E., and McFadden, B. A.: Use of membrane filters in the measurement of biological incorporation of radioactive isotopes. *J. Bact.*, 1956, 71:123.
- Bard, R. C., and McClung, L. S.: Preparation of Media. *Manual of Methods. Pure Culture Study of Bacteria*, 1950, 18:Leaflet II. Biotech Publications, Geneva, N. Y.
- Berman, L., and Stulberg, C. S.: Eight culture strains (Detroit) of human epithelial-like cells. *Proc. Soc. Exp. Biol. and Med.*, 1956, 92:730.
- Bieseke, J. J.: Tissue culture and cancer. *Sci. Am.*, 1956, 195:50.
- Braun, W., and Kelsh, J.: Improved method for cultivation of *Brucella* from the blood. *Proc. Soc. Exp. Biol. and Med.*, 1954, 85:154.
- Brochure, Difco Manual of Dehydrated Culture Media and Reagents. Difco Laboratories, Inc., Detroit, Mich. 9th ed., 1953.
- Brochure, Products for the Microbiological Laboratory. 4th ed. Baltimore Biological Laboratory, Baltimore 18, Md., 1956.
- Brueck, J. W., and Buddingh, G. J.: Isolation of *M. tuberculosis* by inoculation of the yolk sac of embryonated eggs. *Proc. Soc. Exp. Biol. and Med.*, 1952, 80:589.
- Buddingh, G. J.: Chick embryo techniques. In Rivers, T. M.: *Viral and Rickettsial Infections of Man*. 2nd ed. J. B. Lippincott Co., Philadelphia. 1952.
- Cahnock, R. M.: Cytopathogenic effect of Newcastle virus in monkey kidney cultures and interference with poliomyelitis viruses. *Proc. Soc. Exp. Biol. and Med.*, 1955, 89:379.
- Cheldelin, V. H., and King, T. E.: Nutrition of microorganisms. *Ann. Rev. Microbiol.*, 1953, 7:113.
- Cox, H. R.: Growth of viruses and rickettsiae in the developing chick embryo. *Ann. New York Acad. Sci.*, 1952, 55:236.
- Delwiche, E. A.: Metabolism of microorganisms. *Ann. Rev. Microbiol.*, 1955, 9:45.
- Eagle, H.: Nutrition needs of mammalian cells in tissue culture. *Science*, 1955, 122:501.
- Eagle, H., Oyama, V. L., Levy, M., and Freeman, A.: Myo-inositol as an essential growth factor for normal and malignant human cells in tissue culture. *Science*, 1956, 123:845.
- Editorial: The role of the molecular filter membrane in the field of Public Health. *Am. J. Pub. Health*, 1953, 43:766.
- Ehrlich, R.: Technique for microscopic count of microorganisms directly on membrane filters. *J. Bact.*, 1955, 70:265.
- Enders, J. F.: Cytopathology of virus infections. *Ann. Rev. Microbiol.*, 1954, 8:473.
- Evans, C. A., Chambers, V. C., Smith, W. N., and Byatt, P. H.: Growth of neurotropic viruses in extraneural tissues. *J. Inf. Dis.*, 1954, 94:273.
- Evenson, M. A., and Gerhardt, P.: Nutrition of brucellae: utilization of iron, magnesium, and manganese for growth. *Proc. Soc. Exp. Biol. and Med.*, 1955, 89:678.
- Fry, B. A., and Peel, J. L., Eds.: *Symposium on Autotrophic Microorganisms*, Soc. Gen. Microbiology. Cambridge University Press, New York, 1954.
- Gay, K., and Damon, S. R.: A yolk sac technique for the routine isolation of *Brucella*. *Public Health Reports*, 1951, 66:1204.

- Geldreich, E. W., Kabler, P. W., Jeter, H. L., and Clark, H. F.: A delayed incubation membrane filter test for coliform bacteria in water. *Am. J. Pub. Health*, 1955, 45:1462.
- Gibbs, M., Sokatch, J. T., and Gunsalus, I. C.: Product labeling of glucose-1-C<sup>14</sup> fermentation by homofermentative and heterofermentative lactic acid bacteria. *J. Bact.*, 1955, 70:573.
- Hendlin, D.: The nutrition of microorganisms. *Ann. Rev. Microbiol.*, 1954, 8:47.
- Hotta, S., and Evans, C. A.: Cultivation of mouse-adapted dengue virus (Type I) in rhesus monkey tissue culture. *J. Inf. Dis.*, 1956, 98:89.
- Ikawa, M., and O'Barr, J. S.: The nature of some growth stimulating substances for *Lactobacillus delbrueckii*. *J. Bact.*, 1956, 71:401.
- Johnson, R. B.: Factors influencing the growth of *Shigella dysenteriae* I in a synthetic medium. *J. Bact.*, 1954, 68:604.
- Jordan, W. S.: Human nasal cells in continuous culture: I and II. *Proc. Soc. Exp. Biol. and Med.*, 1956, 92:867 and 872.
- Kamen, M. D.: *Radioactive Tracers in Biology*. Academic Press, Inc., New York, 1951.
- Kingsbury, J. M., and Barghoorn, E. S.: Silica gel as a microbiological medium: potentialities and a new method of preparation. *Appl. Micro.*, 1954, 2:5.
- Kuchler, R. J., and Merchant, D. J.: Propagation of strain L (Earle) cells in agitated fluid suspension. *Proc. Soc. Exp. Biol. and Med.*, 1956, 92:803.
- Kuzdas, C. D., and Morse, E. V.: A selective medium for the isolation of brucellae from contaminated materials. *J. Bact.*, 1953, 66:501.
- Lamanna, C., and Mallette, M. F.: *Basic Bacteriology*. Williams & Wilkins Co., Baltimore, Md., 1953.
- Lees, H.: *Biochemistry of Autotrophic Bacteria*. Butterworth's Scientific Publications, London, 1955.
- Lennette, E. H.: Symposium on newer knowledge of viral and rickettsial diseases. *Am. J. Trop. Med. and Hyg.*, 1956, 5:419.
- Lichstein, H. C.: Metabolism of Microorganisms. *Ann. Rev. Microbiol.*, 1952, 6:1.
- Mair, N. S.: A selective medium for the isolation of *Brucella abortus* from herd samples of milk. *Monthly Bull. Min. of Health, Public Health Lab. Serv. (London)*, 1955, 14:184.
- Marcus, S.: Disposable Petri-type dish. *Science*, 1955, 122:762.
- Melnick, J. L.: Application of tissue culture methods to epidemiological studies of poliomyelitis. *Am. J. Pub. Health*, 1954, 44:571.
- Morgante, O., and Murray, E. G. D.: The isolation of *Mycobacterium tuberculosis* by filtration techniques from cerebrospinal fluid. *Canad. J. Micro.*, 1955, 1:331.
- Oginsky, E. L., and Umbreit, W. W.: *An Introduction to Bacterial Physiology*. W. H. Freeman and Co., San Francisco, Calif., 1954.
- O'Kane, D. J.: Metabolism of carbohydrates and related compounds. *Ann. Rev. Microbiol.*, 1956, 10:275.
- Oppenheimer, C. H.: The membrane filter in marine microbiology. *J. Bact.*, 1952, 64:783.
- Pappenheimer, A. M., Mueller, J. H., and Cohen, S.: Production of potent diphtheria toxin on a medium of chemically defined composition. *Proc. Soc. Exp. Biol. and Med.*, 1937, 36:795.
- Proom, H., and Knight, B. C. J. G.: The minimal nutritional requirements of some species in the genus *Bacillus*. *J. Gen. Micro.*, 1955, 13:474.
- Richards, C. W., and Krabek, W. B.: Visualizing microorganisms on membrane filter surface. *J. Bact.*, 1954, 67:613.
- Rivers, T. M., Ward, S. M., and Baird, R. D.: Amount and duration of immunity induced by intradermal inoculation of cultured vaccine virus. *J. Exper. Med.*, 1939, 69:857.
- Robineaux, R., Barski, G., and Endo, M.: Phase contrast cinematography of cellular lesion produced by poliomyelitis virus in vitro. *Proc. Soc. Exp. Biol. and Med.*, 1955, 88:57.
- Slanetz, L. W., and Bartley, C. H.: Evaluation of membrane filters for the determination of numbers of coliform bacteria in water. *Appl. Micro.*, 1955, 3:46.
- Slanetz, L. W., Bent, D. F., and Bartley, C. H.: Use of the membrane filter technique to enumerate enterococci in water. *Pub. Health Reports*, 1955, 70:67.
- Stadtman, E. R., and Stadtman, T. C.: Metabolism of microorganisms. *Ann. Rev. Microbiol.*, 1953, 7:143.
- Stokstad, E. L. R., Broquist, H. P., and Sloane, N. H.: Nutrition of microorganisms. *Ann. Rev. Microbiol.*, 1955, 9:111.

- Sykes, G., Editor: *Constituents of Bacteriological Culture Media*. Cambridge University Press, New York, 1956.
- Takemoto, K. K., et al.: Primary isolation of influenza A, B, and C viruses in monkey kidney tissue cultures. *Proc. Soc. Exp. Biol. and Med.*, 1955, 89:308.
- The Staff of the Tissue Culture Course, 1949-1953, Cooperstown, N. Y. *An Introduction to Cell and Tissue Culture*. Burgess Publishing Co., Minneapolis 15, Minn.
- Thomas, H. A., and Woodward, R. L.: Estimation of coliform density by the membrane filter and the fermentation tube methods. *Am. J. Pub. Health*, 1955, 45:1431.
- Thorne, C. B.: Metabolism of nitrogenous compounds. *Ann. Rev. Microbiol.*, 1956, 10:329.
- Tidwell, W. L., and Gee, L. L.: Use of membrane filter in blood cultures. *Proc. Soc. Exp. Biol. and Med.*, 1955, 88:561.
- Tomlinson, A. J. H., and Mitchell, E. R.: Isolation of poliomyelitis virus in a routine bacteriological laboratory. *Monthly Bull., Min. of Health and Pub. Health Lab. Serv.*, London, 1956, 15:53.
- Vallee, B. L.: The metabolic role of zinc. *J.A.M.A.*, 1956, 162:1053.
- Waksman, S. A. Ed.: *Perspectives and Horizons in Microbiology*. Rutgers Univ. Press, New Brunswick, N. J., 1955.
- Wayne, L. G.: Cultivation and visualization of mycobacteria on molecular filter membranes. *J. Bact.*, 1955, 69:92.
- Weinberg, E.: Selective inhibition of microbial growth by incorporation of triphenyltetrazolium-Cl in culture media. *J. Bact.*, 1953, 66:241.
- Werkman, C. H., and Wilson, P. W.: *Bacterial Physiology*. Academic Press, New York, 1951.
- White, P. R.: *The Cultivation of Animal and Plant Cells*. The Ronald Press Co., New York, 1954.
- Wright, L. D.: Nutrition of bacteria and fungi. *Ann. Rev. Microbiol.*, 1956, 10:141.
- Zitcer, E. M., Cailleau, R., and Kirk, P. K.: Serial cultivation of normal human embryonic cells directly on glass. *Science*, 1954, 120:507.
- ZoBell, C. E.: The effect of solid surfaces upon bacterial activity. *J. Bact.*, 1943, 46:39.

## The Growth of Bacteria

### REPRODUCTION

**Binary Fission.** The most obvious means of reproduction by organisms of the class Schizomycetes is *transverse* division of each cell into two approximately *equal* cells. This is called simple or binary fission (Fig. 14-1).

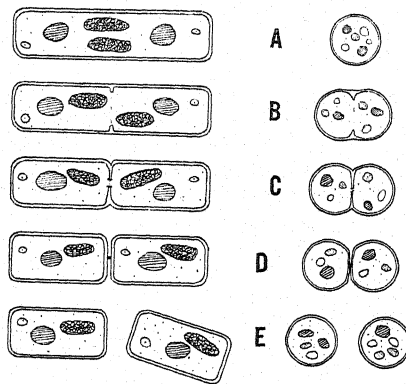
**Bacterial Multiplication by Means Other than Binary Fission.** Other forms of multiplication of bacteria, especially by means of intracellular bodies, variously called gonidia, microcysts and regenerative granules, have been described. In these types of reproduction, multiplication may be regarded as resulting from intramural divisions of the cell material so that all of the new organisms are contained within the original cell wall and are necessarily very tiny. Rupture of the cell wall liberates the tiny granules. There is no convincing evidence that such minute bodies occur in relation to the reproduction of bacteria unless the formation of L forms of PPLO in some species (Chapter 8) be so interpreted. Some spirilla are said to multiply by formation of many "baby" spirilla inside a parent cell.

In attempting to demonstrate *nuclei*, *gonidia*, etc., one is very apt to be misled by the presence of volutin granules, fat droplets and, in dead, dying or artificially mounted cells, of globules of coagulated protein. Diffraction and refractive effects of minute granules are much enhanced in bacteria due to the acute curvature of their minute surfaces. These optical effects are undoubtedly a source of some error in such studies. The electron microscope has yielded exceedingly valuable information in this field. Yet the existence and nature of reproduction other than binary fission is still not clear.

**Sexual Multiplication of Bacteria.** The question of the existence of sexes among bacteria is a much debated one. Evidence based on what appears to be mitosis, meiosis, crossing over and recombination of genes and segregation of characters is very persuasive but, again, is open to question and other possible interpretation.

Protrusions and zygospor-like and nucleus-like bodies undoubtedly appear, but their role in sexual reproduction is not firmly established. They, or appearances very much like them, can be induced by various artificial agents: irradiation with ultraviolet light, colchicine, excess salt, and others. Although many persuasive arguments have been advanced one could wish for more conclusive proof. From an evolutionary point of view, sex in bacteria would

Fig. 14-1. Diagrammatic representation of binary fission as it occurs in rod-shaped and in spherical bacteria. *A*, mature cells ready to divide. *B*, early stage in formation of a wall dividing the two new cells. Note that the intracellular matter (nuclear or hereditary material, gene-like structures, enzymic granules, food vacuoles, and the like) are approximately evenly divided between the two daughter-cells-to-be. *C*, further development of new cell walls and division of all essential materials; the two cytoplasms are still connected. *D*, almost complete separation. The connection between the cytoplasms is now very tenuous, constituting what is sometimes called a *plasmodesma*. By means of this structure cells often cling together in long chains after fission; forming *streptobacilli* in the case of rod forms, *streptococci* in the case of spherical bacteria. In *E*, fission and separation are complete; new intracellular developments are occurring. In some species the entire process of growth, maturation and fission is completed every 20 to 30 minutes.



be an anachronism. According to Coulter, "... sex is not an essential feature of reproduction [in the plant kingdom]. Historically it was the last method of reproduction attained among plants, and when it appeared it did not replace older methods [fission] but was added to them . . . In the evolutionary sequence of plant groups the sexual cells appear for the first time far above the most primitive known plants . . . [bacteria] . . . The beginnings of sexual cells are seen among both the green algae [as *Ulothrix*] and the brown algae. . . ."

#### BACTERIAL "POPULATIONS"

Regardless of the problem of the sexual life of bacteria, the little creatures find no difficulty in multiplying under optimal conditions. If left to their own devices they can, within 24 hours or so, produce inside of a thimble-full of broth a population of astronomical proportions, running into thousands of millions and billions. You may think it queer, like oxidation without oxygen (Chapt. 13), life in a vacuum at 400° below zero F (Chapt. 12), and reproduction without sex, but we can count these billions with reasonable accuracy, in duplicate or quadruplicate, quite easily within a few hours or *even minutes*! If you want to know more, read on.

The numbers (or "populations") of bacteria present in various natural materials such as soil or river water vary constantly due to changing conditions. In an "ideal" situation, however, such as a pure laboratory culture under uniform, optimal physical and chemical conditions, the numbers of organisms present vary in a perfectly regular and predictable way in accord with general biological laws.

As an illustration we may select a culture flask containing 50 ml of sterile infusion broth at 35° C.



**The Enumeration of Bacteria.** Let us introduce, by means of a sterile pipette, a drop of fluid containing about 10 cells of the common, harmless bacterium of the intestinal tract: *Escherichia coli*. Let us assume that these 10 cells are from an inactive or "dormant" stock culture held for weeks in the refrigerator. The new culture is held at 35° C. The problem before us is to measure the population at regular intervals by counting the numbers of living cells present. The numbers present at the different periods are then plotted in relation to time, and a "growth curve" is obtained. Enumeration may be done by several methods, none of which is exact.

One, the hemacytometer or counting chamber method, is to place a minute drop of the fluid in a tiny, shallow, rectangular, glass vessel (hemacytometer) partitioned off by ridges into regular cubical chambers of exactly known volume. By counting the individual cells in each chamber under a microscope, the numbers of organisms per ml. may be computed. This is a *total count* of live and dead organisms.

Another procedure is to smear an exact volume of the culture over an exact area on a slide, stain with methylene blue and count the organisms in a known portion of the total area. Knowing from previous measurements (by means of a stage micrometer) the diameter of the microscopic field, it is easy to calculate the numbers of organisms per ml. of culture. This, also is a total count since no distinction is readily made between living and dead organisms.

Still another method is to place a fixed volume, say 10 ml, of the culture in a kind of test tube having a narrow, hollow, cylindrical column projecting from the bottom and graduated in mm. The organisms are packed into the column by centrifugation at a standard speed and for exactly measured time and their total volume read on the graduated scale. From a knowledge of the average volume of the individual cells an estimation of numbers is possible. This also is a total estimate.

If 1 ml of blood and 1 ml of culture are well mixed, and a stained smear of the mixture prepared, an *estimate* of the numbers of bacteria may be obtained by counting both blood and bacterial cells in a certain number of fields and noting their relative proportions. Knowing that male human blood contains about 5 million erythrocytes per cubic millimeter an estimate of the numbers of bacteria is merely a matter of arithmetic. This is a total estimate.

A widely used technique is turbidometric, i.e., measurement of the turbidity in the fluid due to the accumulation of cells in it. A measured volume of the culture is placed in a special, clear, glass tube of known diameter. This is interposed between a unit source of light and a photoelectric unit which is attached to a galvanometer. The reading on the galvanometer depends on the interference with the passage of light from the unit source, due to the cells suspended in the tube. The method is subject to errors due to variation in size and shape and clumping of cells, as well as to different degrees of translucency of various species and other materials in cultures. However, the method is one of the quickest and simplest and is reasonably accurate. It is a *total estimate*. Turbidity readings may be standardized in terms of numbers of cells by hemacytometer counts.

The method of serial dilutions is widely used to estimate numbers of bacteria in water, milk, various foods, soil, blood, etc. Into tubes of broth are placed 1-ml quantities of the sample (of, let us say, milk), diluted in decimal, 4-fold, 2-fold or other convenient series. After incubation of the tubes of broth numbers of organisms are recorded by noting presence or absence of growth. For example, in a 10-fold dilution series suppose there is growth in the tube which received the 1:1000 dilution but no growth in the tube receiving the 1:10,000 dilution. Then there were between 1,000 and 10,000 organisms per ml of the sample of milk tested. This is spoken of as the *indicated number* (the reciprocal of the highest

dilution showing growth). But it is not a very exact estimate. It measures *only live cells* viable (capable of growth) under the conditions provided.

**MOST PROBABLE NUMBER.** In the above example there *must be* 1,000 organisms but there *may be*, theoretically, any number up to 9,999 per ml in the sample. What is the true number? This cannot be stated. However, mathematicians have shown that the number *most probably* present may be calculated if the results from duplicate or triplicate simultaneous determinations are known. They have prepared tables showing the *most probable number* indicated by all possible combinations of results in such series. These are much used in sanitary examination of water. Tables are found in "Standard Methods for the Examination of Water, Sewage and Industrial Wastes"\* with directions for use. It is to be borne in mind that these are *most probable* numbers; not necessarily *exact* numbers.

**The Colony Count.** The colony count is a widely applicable procedure used daily for determining approximate numbers of bacteria in milk, water, soil, foods, etc.

Continuing the examination of our flask of culture inoculated with *Escherichia coli* we may, at any desired moment, withdraw exactly 1 ml of the culture from the flask with a sterile measuring pipette and transfer it to a sterile Petri dish. Immediately afterward, about 15 ml of nutrient agar of appropriate composition, previously melted (and cooled to about 40° C so as not to kill the bacteria), are poured into the dish. The culture is thoroughly mixed with the still-fluid agar by a gentle horizontal rotation of the dish. In a few minutes the agar will have solidified. This plate culture is held in an incubator at about 35° C for twenty-four hours and is then examined for the presence of *colonies*,† distributed throughout the agar. Each of these represents, *theoretically*, the progeny of a single cell which was in the original inoculum and which was imprisoned in (or on) the agar at that point. Actually several organisms, if stuck together in a clump, will give rise to only a single colony. The colony count, therefore, does not give a wholly accurate enumeration of the live, *individual* cells present in the material under investigation. However, the errors in the plating method are known, in a general way, and within its limitations the plate count is one of our most useful means of enumerating bacteria. It is very widely used and should be fully understood at this point. It measures only organisms viable under the conditions of growth (i.e., kind of medium, temperature, etc.) provided.

The counting of colonies in agar after only a few hours incubation is greatly facilitated by the use of a stereoscopic microscope with both direct and indirect illumination.

As the number of bacteria in the sample increases toward several hundreds or thousands per ml, the 1 ml of material removed for the plate count is diluted so that plates are obtained which show only about 50 to 300 colonies. This is easy after a little experience. It avoids lethal crowding of the colonies and separates the colonies so that counting is easier. The number of colonies, multiplied by the dilution, gives the "number per ml."

**Determination of Growth Curve.** Let us suppose that we make plate counts on our culture of *Escherichia coli* every two hours at first, and plot the logarithms of the numbers of colonies (roughly, live organisms per ml) against time. If we were to plot *actual* numbers instead of logarithms, we should

\* American Public Health Association, New York, 10th ed., 1955.

† It is often desirable to examine the plate by means of a special illumination and a 2 × or 3 × magnifying glass so as to make even the smallest colonies visible.

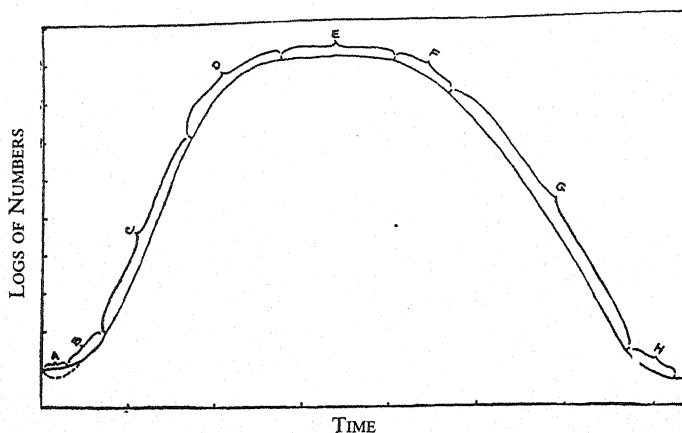


Fig. 14-2. Growth curve of hypothetical culture of any microorganisms under optimal conditions of growth. For explanation see text.

need a *very* large sheet of paper because the numbers often run into billions per ml. We may continue making plate counts until no further significant changes in numbers occur. At the end of this time a curve will have been obtained which will look somewhat like that seen in Figure 14-2.

A totally different type of curve would be obtained if we were to count the bacteria in the fluid by means of one of the *total* count methods described above. This is because many of the bacteria die in the culture during the 24 hours of incubation and, while appearing in the total count, cannot produce growth or colonies in the dilution-tube series or the colony count in Petri plates since these are *viable* counts. A curve showing total counts as compared with viable counts is seen in Figure 14-3.

**Phases of the Growth Curve.** The curve shown in Figure 14-2 has several portions which deserve discussion. These are shown by brackets and labels in the figure.

**INITIAL PHASES.** Portion A, usually called the *latent* or *initial stationary phase*, represents a period during which the dormant organisms are probably imbibing water and/or becoming adjusted to the new environment, much as might occur when a dormant tree is set out in the spring. The exact details of this "awakening" or "reanimation" process are not known. There is no immediate growth (increase in numbers). The dotted line indicates that some few of the cells may actually die off during this period, only the more vigorous going on to multiplication.

**PHASE OF ACCELERATED GROWTH.** Once growth begins it is soon manifested in the rising inflection of portion B which is properly called the *phase of accelerated growth* but is often referred to as the *lag phase*. The first two phases together also are often called the lag phase. During this early period, when fission is slow, the size of the cells is large: hear the maximum for the species. This probably represents inhibition of water with consequent swelling, and the beginning of metabolic activity before fission has occurred.

**CELL WALL AND FISSION.** It is not unlikely, though not proven, that the cell walls of the old or dormant cells of the inoculum are thicker and less

elastic and permeable than those of very young, actively-multiplying cells; the older, mature cells possibly becoming somewhat like the arthrospores of higher fungi. Certainly, mature and dormant bacterial cells are well known to be much more resistant to most deleterious influences: heat, disinfectants, radiations, antibiotics, phage, etc., than young, reproducing cells, which are notoriously vulnerable to such agents.

This thicker wall, if it actually occurs, might interfere with active fission. After inhibition of sufficient water the osmotic pressure within the cell may help initiate fission. Thus, growth and synthesis of protoplasm probably begin very quickly but fission, and consequent increase in numbers, do not become evident so soon.

**INTERFERENCE WITH FISSION.** Fission of growing bacteria is readily interfered with by numerous factors, some known, others obscure. The effect is especially obvious in bacilli. In rod-shaped organisms growth without fission (or at least without division into separate cells) forms long, tubular filaments. This suggests a radially rigid tube with open, or less rigid, ends where growth occurs.

Fission is retarded by the presence of surface-tension reducents, by  $Mg^{++}$  deficiency, by colchicine, by ultraviolet irradiation, by certain variations inducing rough (or R) variants and other factors. The development of filamentous cells is accompanied by profound physiological alterations, some known (e.g., loss of capsule formation and changed antigen content), others not known.

During the phase of accelerated growth the time required for each cell to divide gradually decreases, and fission occurs more and more rapidly as the organisms become adapted to the new culture medium.

**LOGARITHMIC PHASE.** As growth continues the cells reach their maximum rate of fission, which may become so rapid that the number of organisms doubles every twenty to thirty minutes. The average size of the cells is at its minimum for the species during this time. It is conceivable that cell walls are thinnest during this period. Fission rate varies greatly with different species and under different conditions of growth. Tubercle bacilli, for example, probably divide only about once a day at the highest rate of growth.

During this period of most active multiplication (C) the logarithms of the

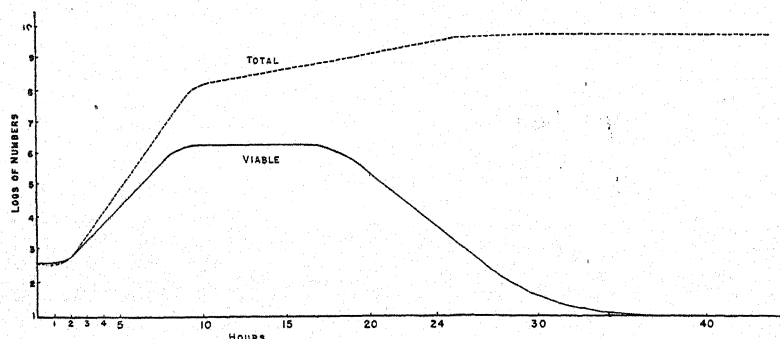


Fig. 14-3. The relation of total to viable counts of bacteria in a pure culture under optimal conditions of growth.

numbers of live organisms counted at short intervals, plotted against time, produce a straight line as shown in Figure 14-2. This period is spoken of as the *phase of logarithmic increase*. Were this to continue uninterrupted, the culture would become a solid mass of bacteria in a few hours. A single cell of *Escherichia coli* allowed to continue such growth for a year or so, would produce a mass weighing more than the sun!

During this phase most of the cells are physiologically young and biologically active. If a subculture is made from the flask to a new flask of warm, sterile broth, growth continues at the logarithmic rate; there is no lag or dormant phase. The lag and dormant phases are reproduced in more or less degree in subcultures made during any other phases of the growth cycle than the logarithmic. The young logarithmic cells are apt to be more easily killed or adversely affected by various inimical agents than older or dormant cells. Biochemical peculiarities used for identification of organisms are also usually most manifest during this period. It is important to remember this, as it has a significant bearing on principles of disinfection, chemotherapy and other phenomena to be discussed.

**PHASE OF NEGATIVE GROWTH ACCELERATION.** Within a few hours after the commencement of the logarithmic phase, the organisms begin to encounter difficulties. Food begins to run out, poisonous waste products accumulate, pH changes, hydrogen acceptors are used up, respiration becomes difficult, and the cells interfere with each other. The rate of fission begins to decline and the organisms die in increasing numbers, so that the increase in number of live cells slows, as shown in the portion of the curve labeled *D* of Figure 14-2. This is spoken of as the *phase of negative growth acceleration*.

A number of workers have tried to prevent the development of this phase by arranging the culture apparatus so that waste products and exhausted medium may be drawn off at regular intervals without removing the bacteria. New, fresh medium is added at the same times. Theoretically, a solid mass of cells should finally result. Actually, growth eventually ceases far short of this, and the population curve develops as usual, with only temporary changes (Fig. 14-4). Evidently, like other creatures, microorganisms must have a certain amount of *space* for the individual to thrive.

**IMMORTALITY (?) OF MICROORGANISMS.** It is interesting to speculate upon the relationships between the age of cells, their multiplication and their continued existence without fission. Let us compare the fate of two cells just produced by the fission of their "parent." Under favorable conditions one undergoes certain unknown physical and chemical changes which are the equivalent of "maturation" and, after a few minutes or hours, undergoes fission like its predecessor. What is the age of the new daughter cells? How can a cell undergo the mysterious changes of maturation and *aging* and yet by the process of fission produce two *young* cells? Theoretically, such creatures never grow "old" or undergo senescence. Are they, by virtue of continual renewal of youth, immortal?

The other of our two original cells fails to reach a state in which fission occurs, and remains intact. What is its physiological age status? If transferred to a new culture it may begin multiplication at once and become "young" or it may die. It may die even if left in the original culture. Does it die of "old age"? The explanation of its fate is quite obscure. It is evident that chronological age and physiological age may be very different matters among microorganisms.

**FINAL PHASES.** Eventually (the time depending on the temperature, the size of the flask and volume of fluid, the composition of the medium and numerous other factors), the number of cells dying balances the rate of increase, and the total *viable* population remains unchanged for a time. The

total count continues to increase, but not as rapidly as at first. This phase, the *maximum stationary phase*, is shown at *E*.

As conditions become more and more inimical to the bacteria, the cells reproduce more slowly, and death overtakes them in ever-increasing numbers, as shown at *F*. This is the phase of accelerated decrease or *accelerated death phase*.

This progresses into the *logarithmic death phase* (*G*), during which decrease in number occurs at a regular, unchanging rate.

Finally, conditions begin to reach an equilibrium such that both rate of death and rate of increase tend to balance each other again at a very low population level, and the *phase of readjustment* (*H*) and a *final dormant phase* are attained. Complete sterility of the culture may not ensue for weeks or months, depending on the kind of organism, whether or not the culture is very acid, etc.

**Factors Affecting Growth Phases.** The form of the growth curve may be affected by many factors. For example, if the culture is suddenly plunged into icewater, the curve at once ceases its upward trend, remains flat for a time and then begins to decline. If held at 22° C instead of 35° C (for *E. coli*) the rise in the positive phases is much less abrupt and much more extended. Other factors such as pH, concentration of food, and so on, have their effects also. If the crowding and toxic growth products in the culture are slightly abated by addition of new food solution during the maximum stationary phase, growth immediately resumes at a logarithmic rate for a while. If a continuous supply of food solution (and dilution of toxic products) is continued, the maximum stationary phase is longer. If food is then discontinued, the phase of logarithmic death sets in very soon.

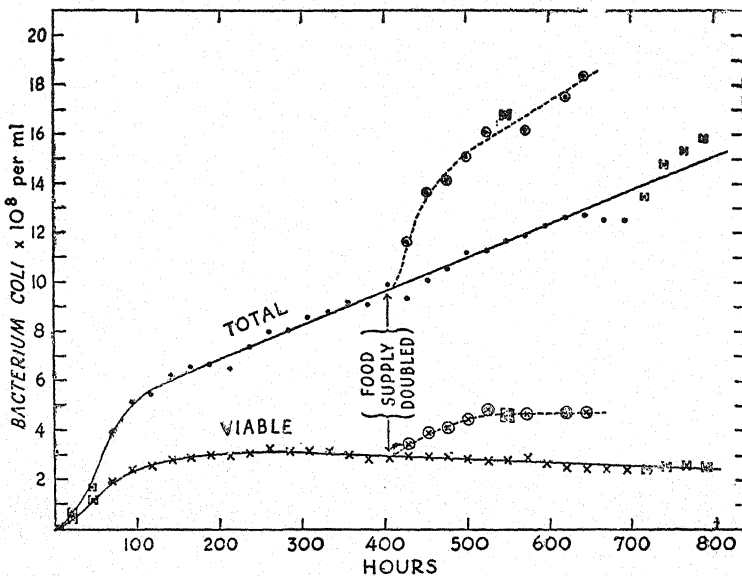


Fig. 14-4. Mean growth curves for *Escherichia coli* on constant food supply of 15.2 mg Difco broth per hr at 35° C, showing effect of renewal of food on growth curve. (Jordan, R. C., and Jacobs, S. E. in *J. Bact.*, vol. 48.)

**Colony Growth.** In the foregoing paragraphs we have considered the development of a microbial population in a flask of *fluid* medium. The surface of a solid medium, like agar, presents a somewhat different situation. We must think of each bacterium in the inoculum as starting a new growth in the very thin film of fluid on the fresh agar surface. Since the new cells\* cannot float and swim very far away from the site of initial growth in the rapidly evaporating film, they start to pile up on one another in a restricted area, sometimes in a pile several thousands of cells high (up to 2 mm). Each heap of cells is a *colony*.

The obvious limitations to colony expansions are the facts that: (a) nutrient solution can diffuse from the agar to the uppermost cells in the colony to only a limited extent; (b) the available supply of nutrient in the agar in the immediate area is soon exhausted; (c) wastes do not readily diffuse away and therefore accumulate in the colony and in the agar beneath. The cells at the top of the heap are obviously at a disadvantage, and soon the upper and central portions of the colony undergo the effects of aging and senescence and other effects of unfavorable nutrition and removal of wastes. Apparent mutations or adaptations to the changed conditions may occur here. The growth of these may appear as new, excrescent colonies on the surface of the old. They are called *secondary colonies*. (See Fig. 16-5.) Active growth may continue at the margin of the colony for some time afterward.

Colonies assume a variety of forms and colors, depending on the species of bacteria, the kind of nutrients in the agar and the conditions of growth. Little is known of the forces which cause one species to form smooth, round, flat colonies, while another produces rough, irregularly shaped colonies with very high conical or domed or "crumbly" form. This is an excellent field for investigation by an ingenious graduate student!

#### REFERENCES

- Buchanan, R. E.: Growth Curves of Bacteria. In *The Newer Knowledge of Bacteriology and Immunology*. University of Chicago Press, Chicago, 1928.
- Clifton, C. E.: A stereoscopic method for counting bacterial colonies. *J. Bact.*, 1955, 69:107.
- Coulter, J. M.: *The Evolution of Sex in Plants*. University of Chicago Press, Chicago, 1914.
- Deibel, R. H., Dowling, M., Niven, C. F., Jr., and Schweigert, B. S.: Filament formation by *Lactobacillus leishmannii* when desoxyribosides replace vitamin B<sub>12</sub> in the growth medium. *J. Bact.*, 1956, 71:255.
- Dickenson, P. B., and MacDonald, K. D.: An electron microscope examination of the initial cell stage in *Streptomyces* spp. *J. Gen. Micr.*, 1955, 13:84.
- Henrici, A. T.: *Morphology, Variation and the Rate of Growth of Bacteria*. Charles C Thomas, Springfield, Ill., 1928.
- Jennison, Marshall W., and Wadsworth, George P.: Evaluation of the errors involved in estimating bacterial numbers by the plating method. *J. Bact.*, 1940, 39:389.
- Jordan, R. C., and Jacobs, S. E.: The effect of temperature on the growth of *B. coli* at pH 7.0 with a constant food supply. *J. Gen. Micr.*, 1947, 1:121.
- Lewis, I. M.: Secondary colonies of bacteria with special reference to *Bacillus mycoides*. *J. Bact.*, 1933, 25:359.
- Novick, A.: Growth of bacteria. *Ann. Rev. Microbiol.*, 1955, 9:99.
- Pease, P.: The gonidial stages in *Spirillum* spp. and *Vibrio* spp. *J. Gen. Micr.*, 1956, 14:672.
- Powell, E. O.: A rapid method for determining the proportion of viable bacteria in a culture. *J. Gen. Micr.*, 1956, 14:153.
- Webb, M.: Effects of magnesium on cellular division in bacteria. *Science*, 1953, 118:607.

\* With the exception of only a few species capable of *swarming*.

- Wenrich, D. H., Lewis, I. F., and Raper, J. R., Editors: Sex in Microorganisms. American Association for the Advancement of Science, Washington, D. C., 1954.
- Werkman, C. H., and Wilson, P. W.: Bacterial Physiology. Academic Press, New York, 1951.
- Winslow, C-E. A.: The Rise and Fall of Bacterial Populations. *In* The Newer Knowledge of Bacteriology and Immunology. University of Chicago Press, Chicago, 1928.
- Winslow, C-E. A., and Walker, H. H.: The earlier phases of the bacterial culture cycle. *Bact. Rev.*, 1939, 3:147.
- Wollman, E. L., and Jacob, F.: Sexuality in bacteria. *Sci. Am.*, 1956, 195:109.



## Variation of Microorganisms

### HEREDITARY MECHANISMS

ALL MICROORGANISMS undergo variations. In bacteria variations are frequently quite obvious: color, form, motility and so on. Other variations are demonstrable only under certain conditions or by special tests: resistance to antimicrobial drugs, virulence, ability to digest certain proteins, and the like. Many of these variations are genetically stable (*heritable*) and appear to depend on alterations in the genetic mechanisms of the microorganisms. In the cells of higher organisms the genetic mechanisms include chromosomes\* and genes organized in demonstrable nuclei.

Some bacteria may have nuclei, with chromosomes. Probably most do not, though the matter is in debate. Viruses, rickettsiae and PPLO do not appear to have definite nuclei. However, all of these microorganisms certainly contain nucleoproteins, and chromatinic bodies more or less suggestive of nuclei and possibly of chromosomes (see Chapt. 10). It is obvious that the *functions* of heredity are carried on by these microorganisms regardless of the *exact form* of the mechanism involved.

**Genes.** It seems probable that microorganisms contain genes, or something very like them. Two distinguishing characters of genes are: (1) self-duplication, and (2) susceptibility to change (mutability). Whether or not grouped in a chromosome or nucleus of the conventional form, genes† determine the distinctive characters of the microorganism, be it the smallest virus or the largest animal or plant: all synthetic (growth) reactions, all respiratory (energy yielding or *exothermic*) reactions, all reproduction, chemical structure, form, motility, color, and, indeed, every conceivable physiological process or attribute of the organism. By duplicating themselves, genes transmit those characters to the daughter microorganism in the process of reproduction, whether by sexual or other means.

**MUTATION.** As long as the genes remain unchanged they continue, like type in a printing press, to make exact replicas of themselves, and so the

\* The term is derived from the Greek words *chroma*, for color, and *soma*, for body. This refers to the fact that when cells are stained with certain dyes for the purpose of studying their structure and emphasizing structural details, the genetic nucleoproteins and related structures take the colors very intensely. They are often spoken of as *chromatin* (color substance). Chromosomes are elongated, rod-like structures in the nucleus.

† The term gene here is extended to include macromolecules of nucleoprotein or nucleic acids having genetic properties.

progeny "breed true" through many generations. If some disturbing influence acts upon them, then the genes are altered and make disturbed (i.e., different) replicas of themselves. One or more of the genes may be destroyed, as often happens under ultraviolet radiation. Then certain properties of the microorganism may be abolished in its progeny as a result. The heritable characters of the progeny will be permanently different from those of the parents. In either case a *genetic mutation* is said to have occurred. The mutant progeny continues to replicate the mutant form, until a new disturbance of genes occurs.

The mutant forms may remain stable and permanent, they may immediately mutate into some new form, or they may revert to the parent form. What occurs is dependent on: (a) the nature of the disturbing agent (which is often called a *mutagenic agent*); (b) the microorganism; and (c) the environment.

Sometimes one gene may control a whole series of successive biochemical reactions produced by a set of individual enzymes. Much depends on whether the enzyme controlled by the gene is in an important "key position" in the biochemistry of the microorganism. Some genes act only to modify, suppress, or enhance the action of other genes. Sometimes a mutation produces effects which are highly beneficial to the mutant organism, sometimes of only indifferent significance under existing conditions, sometimes detrimental and sometimes immediately lethal; i.e., the daughter microorganism is not viable.\* This process of survival of the more fit mutants has been called *natural selection*. Mutation and natural selection are the basis for the origin of new species and are the main spring of organic evolution (Darwin).

The same basic principles and mechanisms apply equally among elephants, whales, mice, men and the most minute microorganisms. Indeed, the more we study and compare, the more likeness we find in fundamental life processes among all creatures, from viruses to man. The chemistry of all is strikingly similar: the digestive, synthetic and respiratory chemical processes; the enzymes; the cell structures; genetic and cell growth mechanisms; responses to environmental influences; and so on and on. Of course, the patterns are modified to fit various types of environment, life history, size, etc., but the basic rules and mechanisms are all obviously derived from the same primitive pattern.

**INDUCED MUTATIONS.** When we produce mutations by some known means under our own control, ultraviolet irradiation for example, we call them *induced mutations*.

**SPONTANEOUS MUTATIONS.** In the process of reproduction genes often become translocated with respect to each other and this definitely affects their action and the characters of the microorganism involved. Further, genes sometimes seem to undergo minor chemical changes within the microorganism, possibly due to molecular collisions, adhesions, etc. Thus, while theoretically progeny inherit parental genic equipment exactly, the genes may be somewhat changed in the very process of multiplication and so the progeny may differ genetically from their progenitors. Thus, genetic mutations may result from intrinsic causes which we cannot control or define. Such mutations are often said to occur "spontaneously."

\* Capable of living.

Spontaneous mutations may be caused also by extrinsic causes: some chemical effect of the surrounding medium, cosmic rays or what you will.

**Plasmagenes.** In addition to genes, certain other character-determining agents almost certainly exist in the *cytoplasm*, apart from the genes localized in the nucleus. They are given various names, a convenient one being *plasmagenes*. Many of these appear to have at least two of the most distinctive properties of genes: self-duplication and mutability. Some plasmagenes may be produced by genes. However, a number are known which gain entrance to the cell from outside, and of which the chemical composition is relatively simple, for example, certain polymerized deoxyribonucleic acids. Some may be extracted and transferred from one cell to another by artificial or natural means. They act as mutagenic agents, which are discussed below.

### MUTAGENIC AGENTS

These are the agents (physical, chemical or biological) that induce mutation. We may group them for convenience as follows:

#### A. Extrinsic mutagens

##### I. Microbiological agents

- a. Macromolecules like certain deoxyribonucleic acids;
- b. viruses, and virus-like factors;
- c. bacteria.

##### II. Radiations, especially ultraviolet and x-rays.

##### III. Injuries or prolonged irritations of various kinds:

These appear to act more obviously on tissue cells in larger animals and to result in cancer, but certain irritants, e.g., nitrogen mustard, are widely studied as mutagenic agents in bacteria.

##### IV. Chemicals, especially methylcholanthrene, arsenic, chromium, urethane, mineral oils, creosote, tar, nitrogen mustard, organic peroxides and $H_2O_2$ , $MnCl_2$ , etc.

##### V. Sex hormones (?)

#### B. Intrinsic Mutagenic Agents

##### I. Recombination of nonidentical chromosomes or portions of chromosomes (in cells possessing chromosomes).

##### II. Translocations of genes within a chromosome or crossing over of genes between chromosomes.

##### III. "Spontaneous" changes in the gene itself; possibly due to molecular impact, adhesions, chemical modifications, etc.

### A. EXTRINSIC MUTAGENS

**1. Microbiological Agents.** Among the extrinsic mutagens those included in Groups a and b are of especial interest at this point.

a. **MACROMOLECULES AND MICROBIAL TRANSFORMATION.** This brings us immediately into a fascinating "never-never land"; the twilight world where heredity and disease, living and non-living, virus, macromolecule and gene are distinguishable with difficulty, like shadowy objects seen under sunlit, shimmering water.

**ENTRAINMENT AND TRANSFORMATION.** As early as 1925, data were published indicating that if one species of bacterium were cultivated in the presence of another (of a rather closely related species) the first would acquire one or more properties of the second. Apparently something passed from one to the other. This was originally spoken of as "*entrainment*." For example, in one

study, a harmless streptococcus from cheese was shown to acquire the property of forming erythrogenic (scarlet fever) toxin when grown in contact with scarlet-fever streptococci. Similarly, in another study the virus of rabbit fibroma\* was changed into myxoma† virus by bringing the fibroma virus into contact with *dead* myxoma virus or *virus-free extracts* thereof. In another study it was found that *extracts of dead*, encapsulated‡ (smooth), type-III pneumococci§ would induce live, non-encapsulated (rough), non-type-specific cells (derived from type-II pneumococci) to change into smooth, type-III pneumococci (see Fig. 15-1). Similar changes have been induced in encapsulated strains of *Escherichia coli* and other bacteria. All of these changes were, in all appearances, biological (genetic) mutations. Genetic changes induced in this way are now spoken of as *transformations*.

THE TRANSFORMING PRINCIPLE. In 1944 the substance in the extracts of smooth, type-III pneumococcus responsible for the change of the rough,

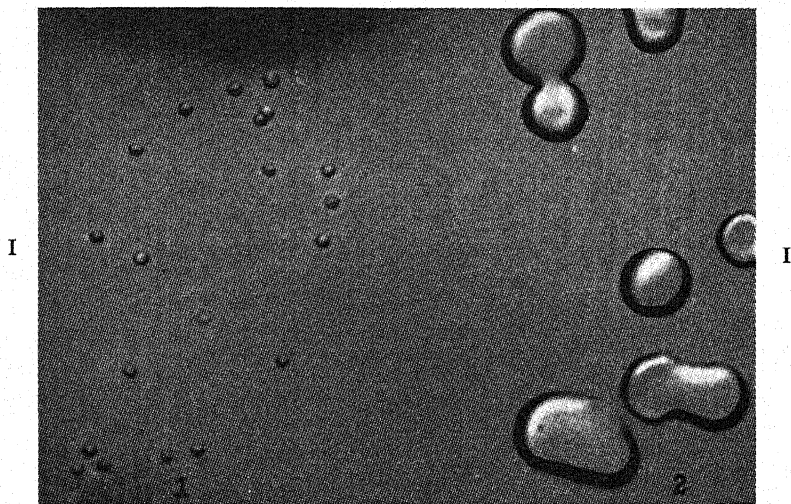


Fig. 15-1. (1) Colonies of R variant of Pneumococcus Type II plated on blood agar from a culture grown in serum broth in the *absence* of the transforming substance ( $\times 3.5$ ). (2) Colonies of the same cells after transformation during growth in the *presence* of active transforming principle isolated from Type III pneumococci. The smooth, glistening, mucoid colonies shown are characteristic of Pneumococcus Type III. (Avery, O. T., MacLeod, Colin M., and McCarty, Maclyn J. *Exper. Med.*, vol. 79.) The photograph was made by Mr. Joseph B. Haulenbeck.

\* A slowly-progressive, not highly-lethal, disease of rabbits resulting in the formation of fibrous tumors of the skin.

† A very malignant, rapidly-fatal, disease of rabbits causing slimy disintegration of rabbit tissues (from Greek *myxa*, for slime).

‡ The capsule of many bacteria has a very distinctive chemical composition in various species and in subdivisions of species called *types*. Without its capsule the bacterium becomes "rough" and loses type characteristics. Encapsulated bacteria are usually found to be in the "smooth" phase.

§ The cause of lobar pneumonia and numerous other infections of the respiratory tract: sinusitis, middle ear, etc. (*Diplococcus pneumoniae*), commonly found in the normal throat.

type-II pneumococci to smooth, type-III pneumococci was found to be a macromolecule of highly polymerized\* deoxyribonucleic acid (DNA).

Thus, the transference of specific, heritable characters from one micro-organism to another is not necessarily dependent on sexual functions, or even on what we ordinarily think of as genes. As exemplified by the transformation of pneumococcal types, here is a substance (DNA) of definite and relatively simple composition, taken from a dead cell, applied to the exterior of a living cell of a different type, which enters the living cell and genetically changes its type to that of the first cell, *permanently*. Is this a mutation? Is it Lamarckian? The chemistry of the altered cell is such that it goes on manufacturing more of the mutagenic substance (DNA) which, in turn, maintains the new type-specificity and *can transmit* it. The DNA becomes a self-replicating (or cell-replicating?) part of a new cell; an artificially transmissible gene?

Does this suggest prophage? Can DNA, transferred from one cell to another, "come alive"? Is such a relationship similar to infection by a virus? Both virus and DNA appear to be self-replicating (or cell-replicated?) genetic material, dependent on a live cell for continued reproduction. Does the DNA enter the nucleus, or a gene, or remain in the cytoplasm? Can *any* cytoplasmic agent exert a genetic effect? It appears so, though the matter is not settled. Such a cytoplasmic genetic agent is called a *plasmagene*.

b. VIRUSES AND VIRUS-LIKE FACTORS: 1. PROPHAGE (PROVIRUS). We have seen, in our discussion of bacteriophage, how DNA enters a 'phage-infected bacterial cell and, as prophage, takes up a position and function as a normal gene or heritable genetic constituent (or a symbiont?) of the cell and confers new, heritable characters upon it. An obvious difference between this phenomenon and the transformation of pneumococci, etc., is that, whereas the pneumococcus-type-transforming DNA is extracted by artificial means from dead pneumococcus cells and is artificially transferred by applying it to the exterior of the transformed cells, the prophage DNA is transferred by a microorganism ('phage) and is placed *within* the transformed cell by the 'phage as part of a *natural* process of *infection*.

2. KAPPA PARTICLES. These are visible particles, about  $1\mu$  in diameter, which contain DNA. They may be introduced, either artificially or during sexual conjugation, into the cytoplasm of a ciliate protozoan, *Paramecium aurelia*. When so introduced these particles apparently reproduce themselves (or are they replicated by the *Paramecium*?).

Like a virus, Kappa can develop only in *living* cells of *Paramecium*. Note that Kappa exist and are transmitted in the *cytoplasm* regularly at each cell division—a type of plasmagene. They induce the cell containing them to undergo an apparent mutation which produces a poisonous agent (a toxin) which is called *paramecin*. This kills other paramecia, not infected with Kappa (but not those containing Kappa). Thus, Kappa acts as a mutagenic agent.

Is Kappa a virus? An errant gene? Is it even alive? At any rate, it is somewhat more than a mere macromolecule of DNA like the pneumococcus

\* A polymerized substance is one in which the unit molecules have combined with each other into large, complex molecules having the same proportionate composition of elements but a multiple of the molecular weight of the unit molecules.

transforming principle. It is a truly cytoplasmic genetic agent, but whether it is a living virus is not yet clear.

There are several known (and probably many still-to-be-discovered) similar cytoplasmic genetic agents, such as the Sigma of *Drosophila*, the mammary gland tumor agent of mice, and chondriosomes of plants (see references).

3. BACTERIOPHAGE AND TRANSDUCTION. In 1951 a discovery of fundamental importance to microbial genetics, and to studies of heredity in general, was made. It was observed that bacteriophage, propagated in toxigenic\* strains of *Corynebacterium diphtheriae*\* and then completely separated from them and later propagated in atoxigenic\* strains of *C. diphtheriae* induced toxigenicity as a mutation in the formerly atoxigenic strains. It was very puzzling, however, to find later that toxigenicity could be induced by 'phage derived from atoxigenic strains. We would like to know exactly how. Perhaps there are "incomplete" genes which can be completed by 'phage.

The phenomenon of transfer of any genetic character by bacteriophage from one bacterium to another is called *transduction*. The mechanism of this transfer is not clear. The idea arose that the 'phage in some way picked up or adsorbed DNA from one bacterial strain and introduced it into another strain. This may be so, but treatment of the 'phage with a powerful enzyme† which destroys DNA did not interfere with the 'phage or the transmission of toxigenicity. However, the DNA might be inside the 'phage particles, protected from the enzyme.

'Phage transduction can transmit numerous other genetic characters in other organisms. Apparently any gene-controlled characteristic may be thus transmitted. In the genus *Salmonella*,‡ for example, various nutrient requirements, enzymic functions, resistance or susceptibility to antibiotics, motility, the chemical makeup of the flagella, and so on, have been transduced. In the genus *Bacillus*§ flagella and motility were transduced by 'phage, from motile *B. cereus* (a harmless saprophyte) to non-motile *B. anthracis* (cause of the disease anthrax). Curiously, in many instances the 'phage which transduced was derived from a non-motile, lysogenic strain of *B. anthracis*. In this last series of tests, DNA-ase applied to 'phage during the transduction completely inhibited the transduction. Presumably the DNA in this example of transduction was on the exterior of the 'phage particles, unlike the DNA in the *C. diphtheriae* toxigenicity transduction or the *Salmonella* transduction, which were not affected by DNA.

c. BACTERIA. One of the most interesting examples of living mutagen is the bacterium (*Agrobacterium tumefaciens*) which causes "crown gall" disease, a malignant growth on the Paris daisy and related plants. Once the growth is initiated it can continue to grow, like a cancer. The initiating bac-

\* *C. diphtheriae* is the bacillus which causes diphtheria (see Chapt. 35). The principal effects of diphtheria on the patient are due to the diphtheria toxin, a poisonous waste product which the toxigenic bacteria secrete whenever they grow in a suitable medium, in a laboratory or a patient's throat. Strains of *C. diphtheriae* are often found which are atoxigenic; that is, they appear never to have had (or to have lost) the power to produce toxin.

† Deoxyribonuclease—(DNAase)

‡ The group containing the bacillus of typhoid fever (gram-negative rods).

§ Gram-positive, spore-forming, aerobic rods.

terium need no longer be present. Apparently a plasmagene-like agent is transferred from bacterium to plant cell.

Under ordinary circumstances the factors continue to "grow" in the cells but can be eliminated by certain experimental procedures so that the cells return to normal. Is such a cure possible in human cancer?

Curiously enough, the plant-tumor-inducing property can be transferred from *Agrobacterium tumefaciens* to several species of closely related, but harmless bacteria by: (1) extracts of infected crown-gall-tumor tissue; (2) killed *A. tumefaciens*; (3) nucleic acid and deoxyribonucleic acid from *A. tumefaciens*. These are all good examples of bacterial transformation.

**Fertilization as a Mutagenic Agent.** The essential feature of sexual fertilization in all forms of life exhibiting sex is at the microscopic, single-cell level. It is the complete fusion of the nucleus of a haploid, male gamete with a haploid, female gamete. A diploid cell results. This, in essence, is as true for the simplest sexually reproducing protozoan or fungus as it is for violets, human beings or whales.\* The essential event is the transference of genetic DNA from male to female gamete. The male DNA enters the genetic mechanism of the female cell, becomes self-replicating and contributes to the genetic character of the resulting diploid cells. Does this suggest infection with a virus? Transduction? Transformation with DNA? Kappa in *Paramecium*, etc.?

We may if we wish, for purposes of argument, regard sex in higher animals as an evolved, improved, very selective, very certain means of transmitting certain, particular, genetic material from one particular cell to another particular cell. On the unicellular level of protozoa, yeasts, etc., simpler and less perfected means exists: simple cell fusion or conjugations, without differentiation of male and female. At the still lower level of bacteria and viruses, the mere carrying of a bit of genetic DNA by any hit-or-miss, catch-as-catch-can means to any "competent" cell in the vicinity appears to suffice: by 'phage, by mere bathing of the cell in a solution of sterile DNA, by agents like Sigma, etc.

**Disease and Mutation.** It may be that some DNA is excessively active, or is associated with protein or cytoplasmic structures that make it incompatible with normal cell development and then it causes disease and we call it a *pathogenic* virus. The line between a pathogenic virus and a plasmagene seems very vague. The true relation of sexual fertilization to living mutagens like 'phage and other viruses, to Kappa, Sigma, DNA, and to (probably) many other DNA-transmitting agents is, of course, not yet clear. That they are all pieces of a grand biological picture-puzzle, which is being gradually put together by researchers, into a beautiful picture of Nature seems an almost inescapable conclusion.

**Genetic Recombination.** One of the principal pieces of evidence favoring the existence of nuclei, chromosomes, genes and sex in bacteria is based on observations interpreted as crossing over and recombination of genes in a particular strain of *Escherichia coli*, as follows. A certain strain of *Escherichia coli*, known as K-12, was irradiated with ultraviolet light to produce mutations. Among the mutant strains were two which had lost certain synthetic

\* In parthenogenesis, an exception common in some invertebrates, an egg cell can develop and grow without fertilization by a sperm cell.

abilities and, to live, *absolutely required* certain nutrient substances which they were able to synthesize before mutation.

As a result of the irradiation, one strain (A) was unable to synthesize biotin and methionine; the other strain (B) threonine and proline. The two nutritionally "deficient" (auxotrophic\*) strains were then *cultivated together* in a medium supplying the required pairs of amino acids: biotin and methionine for A; threonine and proline for B. Thus, both strains were able to grow side by side, providing opportunity for sexual contact. Test for sexual fusion was then made by looking among the progeny of these cells for *recombination* of hereditary characters. From the mixture there were isolated strains capable of synthesizing *all four* amino acids. This suggested that sexual fusion between A and B had occurred, resulting in offspring exhibiting synthetic properties of both A and B. The new, fully-synthesizing (prototrophic†) types (AB) normally occurred too rarely (if at all) as mutants in either of the auxotrophic strains to be detected *before* growth together.

While such data are highly suggestive and stimulating, they may well be susceptible to other interpretations than sexuality and fusion of cells; for example, transduction or transformation by DNA. The matter is one for students of genetics.

**Summary.** In summary we may say that: (1) nucleoprotein (with deoxyribonucleic acid: DNA) appears to be the material basis of heredity. (2) We may postulate that there are several means by which this hereditary material can be transmitted from one receptive cell to another: (a) by sperm during *sexual fertilization* among higher plants and animals; (b) by various means of *nuclear fusion*, with or without perceptible sexual differentiation, as exemplified by conjugation in certain protozoa, yeasts and molds and other fungi (possibly in some bacteria like *E. coli* K 12); (c) by mechanical transfer of DNA as in pneumococcus type *transformation*; (d) by biological transmission of genetic material as seen in 'phage *transduction* of toxigenicity in *C. diphtheriae*; (e) by *latent infection with viruses* as illustrated by lysogeny in bacteria, herpes simplex infection, Sigma, Kappa, chondriosomes, etc.

These processes may be thought of (temporarily at least, for purposes of *argument*) as representing stages in the evolution of the sexual process; each representing improvements in means of transmitting heredity material by developing from the hit-or-miss, catch-as-catch-can processes toward the more and more specific, exclusive and certain methods represented by sperm-ovum mechanisms. There appears to be a gradation in the complexity both of material transmitted and of transmitting agency.

#### REFERENCES

- Austrian, R.: Bacterial transformation reactions. *Bact. Rev.*, 1952, 16:31.  
Belser, W. L., and Bunting, M. I.: Studies on a mechanism providing for genetic transfer in *Serratia marcescens*. *J. Bact.*, 1956, 72:582.  
Braun, A. C.: Plant cancer. *Sci. Am.*, 1952, 7:66.  
Braun, W.: Bacterial Genetics. W. B. Saunders Co., Philadelphia, Pa., 1953.  
Brown, E. R., Cherry, W. B., Moody, M. D., and Gordon, M. A.: The induction of motility in *Bacillus anthracis* by means of bacteriophage lysates. *J. Bact.*, 1955, 69:590.

\* *Auxo* is from a Greek word meaning aided; *trophic* denotes nutrition; hence, auxotrophs require "aided nutrition."

† *Proto* is from a Greek root meaning original or primitive.



- Crick, F. H. C.: The structure of the hereditary material. *Sci. Am.*, 1954, 191:54.
- Davis, B. D.: Bacterial genetics and drug resistance. *Pub. Health Rep.*, 1952, 67:376.
- Demerec, M., Editor: *Advances in Genetics*, VII, 1955; VIII, 1956. Academic Press, Inc., New York.
- Dobzhansky, T.: *Evolution, Genetics, and Man*. John Wiley and Sons, Inc., New York, 1955.
- Dodson, E. O.: *Genetics, The Modern Science of Heredity*. W. B. Saunders Co., Philadelphia, 1956.
- Edwards, P. R., Davis, B. R., and Cherry, W. B.: Transfer of antigens by phage lysates with particular reference to the *l*, *w* antigens of *Salmonella*. *J. Bact.*, 1955, 70:279.
- Frobisher, M., Jr., and Brown, J. H.: Transmissible toxigenicity of streptococci. *Bull. Johns Hopkins Hosp.*, 1927, 41:167.
- Gale, E. F., and Davies, R.: *Adaptation in Microorganisms*, Third Symposium, Soc. General Micro. Cambridge University Press, New York, 1953.
- Glass, B.: Genetic recombination. *Science*, 1954, 120:290.
- Grant, V.: The development of a theory of heredity. *Am. Sci.*, 1956, 44:158.
- Groman, N. B.: Evidence for the active role of bacteriophage in the conversion of nontoxic *Corynebacterium diphtheriae* to toxin production. *J. Bact.*, 1955, 69:9.
- Horowitz, N. H.: The gene. *Sci. Am.*, 1956, 195:79.
- Hotchkiss, R. D., and Weiss, E.: Transformed bacteria. *Sci. Am.*, 1956, 195:48.
- Kaplan, R.: Genetics of microorganisms. *Ann. Rev. Microbiol.*, 1952, 6:49.
- Klein, D. T., and Klein, R. M.: Quantitative aspects of transformation of virulence in *Agrobacterium tumefaciens*. *J. Bact.*, 1956, 72:308.
- Knight, C. A., and Fraser, D.: The mutation of viruses. *Sci. Am.*, 1955, 93:74.
- Lamanna, C., and Mallette, M. F.: *Basic Bacteriology*. Williams & Wilkins Co., Baltimore, Md., 1953.
- Lederberg, J.: Conjugal pairing in *Escherichia coli*. *J. Bact.*, 1956, 71:497.
- Lederberg, J.: Recombination mechanisms in bacteria. *J. Cell. and Comp. Physiol.*, 1955, 45:75.
- Lederberg, J., and Tatum, E. L.: Sex in bacteria: Genetic studies, 1945-1952. *Science*, 1953, 118:169.
- Lindgren, C.: Recombination in bacteria. *Science*, 1956, 123:897.
- Mika, L. A., Braun, W., Ciaccio, E., and Goodlow, R. J.: The nature of the effect of  $\alpha$ -alanine on population changes of *Brucella*. *J. Bact.*, 1954, 68:562.
- Mirsky, A. E.: The chemistry of heredity. *Sci. Am.*, 1953, 188:47.
- Parsons, E. I.: Induction of toxigenicity in non-toxic strains of *C. diphtheriae* with bacteriophages derived from non-toxic strains. *Proc. Soc. Exp. Biol. and Med.*, 1955, 90:91.
- Ravin, A. W.: Infection by viruses and genes. *Am. Sci.*, 1955, 43:468.
- Ryan, F. J.: Evolution observed. *Sci. Am.*, 1953, 189:78.
- Spiegelman, S., and Landman, O. E.: Genetics of microorganisms. *Ann. Rev. Microbiol.*, 1954, 8:181.
- Uetake, H., Nakagawa, T., and Akiba, T.: The relationship of bacteriophage to antigenic changes in Group E *Salmonellas*. *J. Bact.*, 1955, 69:571.
- Wagner, R. P., and Mitchell, H. K.: *Genetics and Metabolism*. John Wiley and Sons, Inc., New York, 1955.
- Waksman, S. A., Ed.: *Perspectives and Horizons in Microbiology*. Rutgers Univ. Press, New Brunswick, N. J., 1955.
- Wenrich, D. H., Lewis, I. F., and Raper, J. R., Editors: *Sex in Microorganisms*. American Association for the Advancement of Science, Washington 5, D. C. 1954.
- Wollman, E. L., and Jacob, F.: Sexuality in bacteria. *Sci. Am.*, 1956, 195:109.
- Wyss, O., and Haas, F. L.: Genetics of Microorganisms. *Ann. Rev. Microbiol.*, 1953, 7:47.
- Zelle, M. R.: Genetics of microorganisms. *Ann. Rev. Microbiol.*, 1955, 9:45.

## Some Methods of Studying Variations; Common Variational Types

**Rates of Mutation.** "Spontaneous" mutation occurs among bacteria at widely varying rates. Take, for example, mutation to 'phage resistance. In an actively growing culture of a 'phage-sensitive bacterium a mutant cell, *resistant* to 'phage, may occur as rarely as 1 cell in  $10^5$ . This is a fairly high mutation rate. In another culture only one mutant cell may be found among  $10^{10}$  cells or more. On the other hand, one culture was reported in which 1.3 to 15 per cent of all live cells were mutants. Mutation rates range commonly around  $10^7$ .

It is evident that unless large numbers of cells are examined mutants are not likely to be found. In a small culture (5 ml), the billions of cells ( $5 \times 10^9$ ) would contain only around 500 mutant cells. The detection and isolation of these 500 mutant cells among the 5 billion is not easy without special techniques.

**Detection and Isolation of Mutants.** Detection of "obvious" mutants such as sporeforming or non-sporeforming is simple. Anyone who knows about culture methods and the resistance of spores to heat can devise a simple laboratory procedure to detect the presence or absence of spore-forming mutants in a culture. Detection of mutants resistant to various unfavorable factors is obviously done by submitting a culture, containing a billion or so of actively growing cells, to graded concentrations of the unfavorable factors such as an antibiotic or disinfectant. Survivors at each level of concentration obviously must be resistant to that concentration. An adaptation of this principle especially for detection and isolation of mutants of graded resistance to antibiotics consists of the gradient plate.

1. **THE GRADIENT PLATE.** In this method one pours a shallow layer of nutrient agar (20 ml) containing an appropriate concentration of the desired antibiotic into a Petri plate and allows it to solidify in a sloping position (Fig. 16-1). When solid, a second 20 ml of agar is poured over the first layer and the plate is held horizontally till it is solid. Owing to the reciprocating gradations in the thickness of the layers of agar, the concentrations of antibiotic on the top surface will be graded from high at one side of the plate to low at the other.

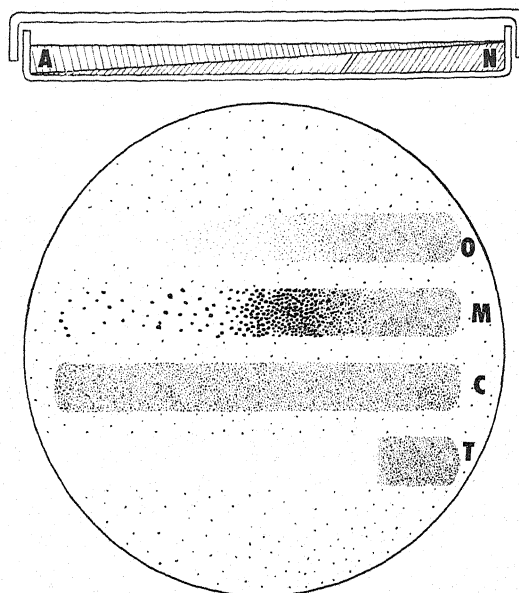


Fig. 16-1. Use of the gradient plate to detect drug-resistant mutants and to measure their degree of resistance. In the Petri plate above, the bottom layer (N) consists of ordinary nutrient agar allowed to solidify in a slanting position. The upper layer (A), poured and allowed to solidify with the plate in a level position, consists of the same sort of agar but with a drug (say, Aureomycin or chlortetracycline) added in measured concentration. The antibiotic diffuses from the upper into the lower layer, leaving a gradient of concentrations of the drug at the surface; greatest at the left of the plate shown above, least at the right. Broth cultures of four different species of bacteria are streaked across the plate, with a sterile brush, parallel with the slope of the agar (in this case, left to right). After incubation it is seen that all organisms grow well at the very lowest concentrations of the drug but react differently in the increasingly greater concentrations. Organism *O* shows moderate resistance of most colonies, a few more than others, but none thriving much more than the majority. Species *M* shows some resistance by most cells but contains some that are wholly resistant and even appear to be stimulated by the drug. Organism *C* shows complete indifference to the drug. Species *T* contains many slightly resistant cells, but all are completely inhibited by exactly the same concentration of the drug. (Adapted from W. Szybalski, in *Science*, 1952, vol. 116.)

A culture is introduced at the area of low concentration and spread thinly toward the area of high concentration. Of the cells which are deposited in the areas of higher concentration only those of higher and higher resistance will grow and form colonies. They are thus revealed as resistant mutants. Their degree of resistance is indicated by their position on the gradient plate.

2. **REPLICA PLATING.** This is really a very clever adaptation of the printers art to bacteriological purposes. One prepares a wooden disk with a flat surface; in diameter about 9 cm (1 cm less than that of a Petri plate). A piece of velvet about 12 cm square is placed smoothly over the surface of the disk, drawn firmly down over the sides and fastened in place with a band. The whole is sterilized.

Let us now select a Petri plate containing nutrient agar, on the surface of which are several hundred colonies. How determine which colony is one of

(a) streptomycin-resistant mutants? (b) "threonineless" auxotrophs? Their appearance in no wise distinguishes them. We could pick a portion of each of the several hundred colonies with a needle and transfer it to (a) a medium containing streptomycin and (b) a medium containing threonine and (c) also one without threonine. If inoculum from any colony grows on the streptomycin-containing medium it is obviously streptomycin-resistant. If it grows on the medium with threonine and not on the same medium minus threonine, it is a threonine-deficient auxotroph. But this entails at least 300 (usually thousands) of inoculations (requiring 28 hours of work per day!) in order to find the one or two mutant colonies among the hundreds of colonies on the plate.

Much time and labor is saved if we press the original plate gently down on the sterile, velvet disk. Each colony leaves a small spot of cells where it touches the velvet. The plate is removed. If, now, we press the surfaces of three sterile plates successively down on the velvet we have, in three motions, "printed" all of the colonies on the original plate in three *replica plates*; (a) one containing medium with streptomycin, (b) a plate with agar containing threonine and (c) a plate containing agar devoid of threonine. The three replica plates are then incubated and colonies develop. Each colony is in its own, readily-determined location on the surfaces of each of the "selective" replica plates (a, b, and c). Only the colony of streptomycin-resistant mutants will grow on the streptomycin-containing plate (a); the threonine-deficient mutant will grow on the threonine plate (b); but not on the plate without threonine (c). The mutant colonies are thus easily identified by their growth and locations on the replica plates (Fig. 16-2).

3. ISOLATION BY PENICILLIN. This method depends on the fact, previously emphasized, that organisms in the logarithmic phase of growth are most susceptible to various deleterious influences, including the action of penicillin.

Keeping this in mind let us irradiate a culture of bacteria with ultraviolet light. Suppose purineless auxotrophs are to be looked for. (As shown in Chapter 12, they are not immediately apparent. Several generations of growth must be allowed before the mutational lag is overcome.) After the proper, post-irradiation incubation, *all* of the cells are removed from the culture medium by centrifugal force. They are resuspended in an inert saline solution in which they have no food and so *do not grow* at all; they are thus "starved" for some hours. Now, to separate the auxotrophs from the prototrophs, penicillin is added to the suspension, along with *just enough* purine-free medium to permit the prototrophs to grow but *not* the purineless, auxotrophic mutants.

As soon as the prototrophs start to grow the penicillin kills them. The still "dormant" auxotrophs are now removed from the penicillin to a purine-containing medium where they grow vigorously and are proudly exhibited to the class.

It might be mentioned that several precautions are necessary in each stage of the procedure, especially against a "joker" called *syntropism*.\*

SYNTROPISM. This little scientific joke is played by the prototrophs in the final stage of the above procedure. At this point the unsuspecting prototrophs are being done to death by feeding them in the presence of penicillin. As they

\* From Greek roots signifying "nourishing each other."

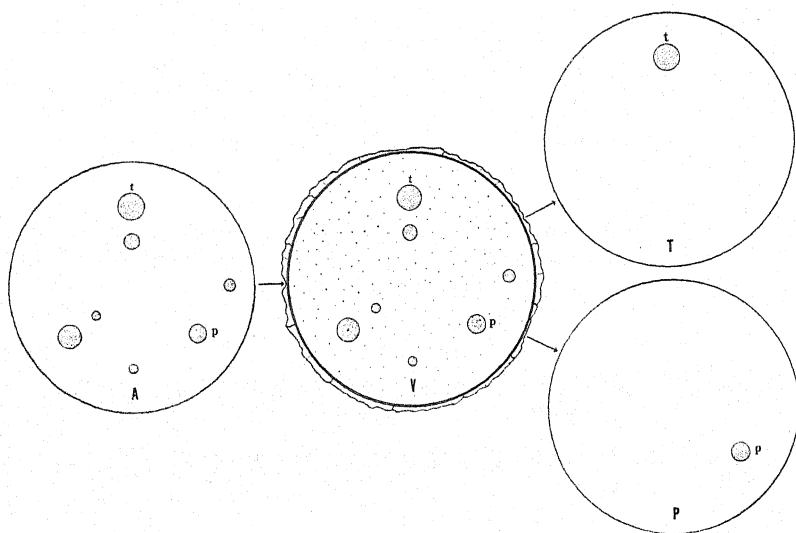


Fig. 16-2. Use of the replica plate for detection of colonies of mutants. Plate *A* contains agar complete in all essential nutrients and free of penicillin. It was inoculated with a culture of a certain species of bacillus. It was then incubated. It is desired to determine which of the colonies (if any) consists (1) of mutants able to grow in the absence of the amino acid, threonine, and (2) of mutants resistant to penicillin. Plate *A* is inverted over, and lightly pressed down upon, the sterile velvet disk *V* and then removed, leaving spots of live organisms in positions corresponding to the positions of the colonies on plate *A*. Immediately thereafter plate *T*, containing sterile agar like that in plate *A* but devoid of threonine, is pressed upon the velvet disk, removed and incubated. Plate *P*, containing agar in all respects like that in plate *A* but with penicillin added in measured concentration, is likewise inoculated by pressing it upon the velvet disk. After incubation, plate *T* has only one colony (*t*) obviously consisting of mutants able to synthesize their own threonine (or able to grow without it). Plate *P* has a colony (*p*) resistant to penicillin in the concentration, at least, in which it exists in plate *P*; perhaps wholly resistant.

start to grow they synthesize just enough purine to start the auxotrophs to growing and so the auxotrophs, too, are killed by the penicillin; a dastardly trick!

It is worth interrupting at this point to emphasize the fact that syntropism is of very great importance in natural ecological relationships. For example, in the soil, one organism *Nitrosomonas* will produce a substance (e.g.,  $\text{NaNO}_2$ ) without which another (*Nitrobacter*) cannot grow. *Nitrobacter* is an autotroph requiring  $\text{NaNO}_2$  as a source of energy. The  $\text{NaNO}_2$  is oxidized to  $\text{NaNO}_3$ , the most valuable and expensive nitrogenous food for agricultural crops. Thus, *Nitrosomonas* nourishes *Nitrobacter* nourishes crops nourishes cattle nourishes man; a quadruple play at home plate!

Syntropism is an important ecological factor operative among bacteria in the soil, the sea, and in any natural, mixed population.

**Adaptation.** Various forms of life living in curious habitats such as ocean ooze, oil wells, hot springs or Great Salt Lake have "adapted" themselves to such situations as though the organisms, aware of the peculiar qualities necessary to inhabit such an environment, actively developed those properties for the specific purpose; an idea based on the doctrine of *entelechy* or teleology,

now obsolete in biology. The inhabitants of such situations are, however, descendants of mutants which, purely by chance, possessed particular physiological properties and, being carried by some purely chance mechanism (wind currents, underground seepage, birds, etc.), were able, because of those peculiar properties, to survive in the environment in which they found themselves. Other cells, not so endowed, perished.

Thus "adaptation" in microorganisms is usually a concise expression of the fact that mutation and natural selection have operated.

**ADAPTIVE ENZYMES.** Very interesting changes, almost suggestive of entelechy, may be induced by cultivating organisms in the presence of certain food substances that they ordinarily do not use. For example, if you continuously cultivate a strain of bacteria not known to ferment lactose, in medium containing lactose, the strain eventually exhibits the property of fermenting the lactose promptly. The cells are found to contain an enzyme *lactase*, which they did not possess in demonstrable amounts before. Removed from contact with the lactose, the power to ferment it may persist, like a mutation, or it may soon disappear. Any enzyme thus appearing in response to an environmental factor is called an *adaptive enzyme*.

The production of adaptive enzyme has been shown to occur in some instances in "resting" vegetative cells; i.e., cells that are alive but not multiplying. This is obviously not selection of a mutant by the environment since there is no growth.

Sometimes it is possible to detect minute amounts of an adaptive enzyme in cells before adaptation; in such instances adaptation seems to be merely an enhancement of an existing though imperceptible function. In other instances, no enzyme is detectable before adaptation. In these cases adaptive enzyme formation seems to represent the induction of a new function. (But failure to find an enzyme does not prove its absence!)

The actual mechanism of adaptive enzyme formation and the nature of the stimulus which starts that mechanism working are not known. That actual synthesis of new enzyme protein often occurs is indicated by the fact that amino nitrogen and an energy source are usually required for adaptive enzymes to develop.

**CONSTITUTIVE ENZYMES.** Any given species of bacterium possesses certain fairly definite and stable properties by means of which we can identify it. Among these properties are form, motility, Gram reaction, and certain inherent enzymic properties such as ability to ferment certain carbohydrates, digest certain proteins, etc. These enzymes are present under all ordinary conditions of growth, whether the substrate on which they act is present or not. The enzymes are part of the permanent constitution of the cell and as such are called *constitutive enzymes*. Perhaps the only difference between a constitutive enzyme and an adaptive enzyme is one of degree of functional activity.

**Mutations Affecting Whole Populations.** Changes which appear to involve whole populations of organisms are in many instances demonstrably due to mutation. For example, in liquid-medium cultures of smooth (S) *Brucella*\* species the amino acid alanine, not initially present in the medium, accumu-

\* Small, gram-negative, non-motile, non-sporeforming rods, the cause of "Bang's disease" (brucellosis or undulant fever) in domestic animals and man.

lates as a waste product of the growing cells. *d*-Alanine (not *l*-alanine) has the effect of suppressing the growth of the smooth (S) cells used for the initial inoculation. But it markedly favors the growth of rough (R) cells. Thus, the whole population appears to change from S to R with age, whereas, actually, the R cells were initially present as mutants in the culture to the extent of about  $1:10^7$  cells. Thus, vast population changes can result from apparently trifling causes initiated by the populations themselves. This is only one illustration of many such alterations.

It is interesting to imagine what might happen if human beings should be now present on the earth, as mutants, who are totally insensitive to the radiation effects of A and H bombs. Radiation-resistant mutants of bacteria occur. Who knows but that you, fair reader, may be radiation-resistant yourself? It is not recommended that anyone stand near an exploding H bomb to ascertain this point about himself. If total war were to occur (perish the thought!) such mutants *could* be the only survivors. This would be mutation and natural selection with a bang!

**Sectors in Colonies.** On a solid agar surface a bacterial colony grows radially. The oldest (and senescent) growth is at the center. The newest (and most active) growth is around the periphery. In the development of a colony the single cell initiating the colony may produce billions of progeny. If a visible type of mutation (for example, production of a bright red pigment) occurs in a single cell during the radial expansion of the colony, a roughly triangular, and visible, sector of red cells appears. The apex of the triangle is toward the center of the colony and is the point at which mutation occurred. The base of the sector is at the periphery of the colony. The cells in the sector are progeny of the mutant which initiated the sector (Fig. 16-3). Another visible type of sector in a colony is produced by loss (or gain) of the property of spore formation. If, in a colony of cells producing spores as they grow, sporeless mutation occurs, the progeny are sporeless and they appear as a translucent sector in the colony (Fig. 16-4).

#### VARIATIONS IN COLONY FORM

**Rough and Smooth Colonies.** These have been described briefly in an earlier chapter. Here we may give a little more detail. Let us consider as an example the colonies produced on infusion agar by a certain strain of gram-positive bacillus. The colonies ordinarily produced by the organism on meat-extract agar are about 2 mm in diameter, gray and translucent. They appear in two different forms: S and R.

**IN THE S FORM** the colonies are perfectly smooth, moist and homogenous, convex, circular and glistening and have regular margins (Fig. 1-6). They are *butyrous* (butter-like) in consistency.

**THE R TYPE OF COLONY** is not glistening but *dull*, and has rough or wrinkled surfaces, and irregular edges. It is dry and crumbly in consistency (Fig. 1-7).

It is commonly observed that the cells in rough colonies form themselves into long tangled filaments on the surface of solid media whereas, in smooth colonies, the cells tend to occur singly.

In broth the rough growth is usually granular or flaky, often growing in a thick scum or pellicle on the surface or settling to the bottom. Long filaments are seen, suggesting a defect in fission, or separation of cells, although growth

obviously occurs. The defect, if it is a defect, may affect only some factor in the cell walls. The smooth growth produces an even turbidity in broth.

The changes in colony form are also intimately related to profound chemical changes in the cells, especially at their surfaces. These in turn are related to defense against outside influences, reactions to electrolytes, virulence, capsule formation and many other characteristics: some visible; some detectable only by selective methods like replica plating; others unknown or not clearly understood.

Practically all species of bacteria vary in this way. There are often intermediate degrees of "roughness" or "smoothness." As suggested in Chapters 3 and 4, this *may* be related to a haploid-diploid phenomenon, or to a dimorphic phenomenon in some molds.

In many species the change from S to R or from R to S is readily induced by any of several stimuli and often occurs "spontaneously." It is thought by some that the alternation in form is not an oscillation between the two ( $R \leftrightarrow S$ ) but a series of progressive mutations  $R \rightarrow S \rightarrow R \rightarrow S$ . The exact facts remain to be revealed.

Frequently S forms seem to represent a reaction to unfavorable environmental factors, though this is not necessarily always so. Contact with disinfectants, certain salts, etc., may stimulate the appearance of S forms. Among bacteria capable of infecting animal tissues, the appearance of the S form is favored by contact with the infected host which resists the infection. Virulence is thus commonly associated with the S form, and this, in turn, with the formation of a visible capsule or with analogous defensive modifications in the surface of the organisms.

These surface alterations, in turn, affect the colloidal properties of the cells and hence

Fig. 16-3. Colonies of one species of bacterium growing on the same agar plate, originating from the same inoculum. Variation between white pigmentation (light) and non-pigmentation or transparent (dark) is clearly evident. Clearly shown also is the appearance of mutants during the development of each type of colony, with resulting pigmented (or non-pigmented), triangular sector in the colony. (Photo courtesy of Dr. Mary Barber, in *J. Gen. Micr.*, 1955, vol. 13.)

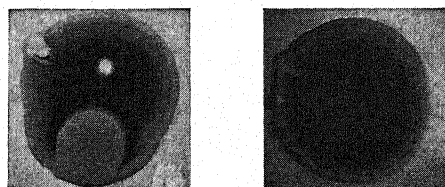
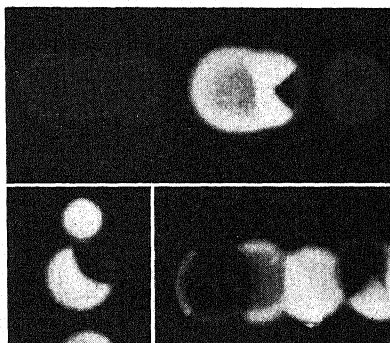


Fig. 16-4. Colonies of a species of spore-forming aerobic bacillus showing sectors (light areas) of nonsporulating variants of the same species. (Photographs courtesy of Dr. J. Howard Brown.) ( $\times$  about 15.)



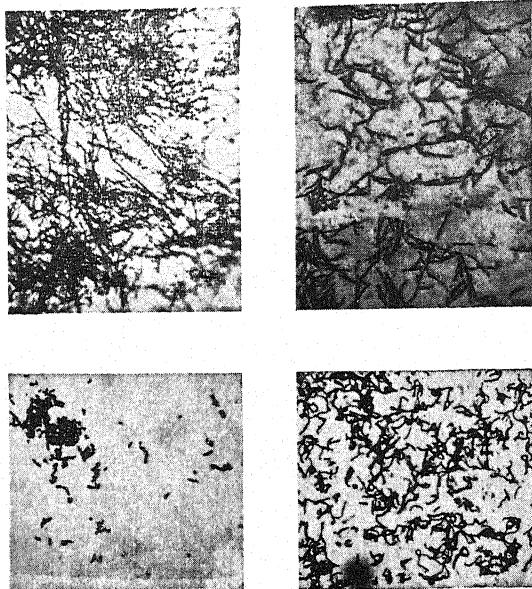


Fig. 16-5. Different morphological types assumed by a single kind of organism ( $\times 900$ ).

their immunologic\* and antigenic† properties (since these are all largely surface phenomena). These alterations in the antigenic properties of organisms are of the utmost importance. Whole systems of results may depend on whether S or R forms are being used.

When S forms of bacteria are cultivated on artificial media in pure culture, removed from competition with other bacteria that occur in natural environments like the soil or throat, under wholly uniform, benign and favorable conditions, they frequently tend to lose their virulence and their protective surface structures and revert to the saprophytic, or possibly less highly differentiated, R form. There are exceptions to this, but in artificial cultures it is common to observe this transition.

Other differences between R and S variants are frequently noted. For example, in the spore-forming organism referred to above, colonies are frequently observed having sectors of non-sporulating cells (Fig. 16-4). By means of a very fine needle and an instrument called a micro-manipulator, these two types of cells (sporulating and non-sporulating) can be separated in pure culture. It was found that a single cell of either type can produce the other, in either the R or S form. Thus, sex is not involved. No reason has been found to account for the variations. They seemed to occur spontaneously. Loss of spore formation is a common observation. In addition to this, cells of the organism in different colonies, often of the same type, varied so widely in size and form as to be unrecognizable as the same organism (Fig. 16-5).

Changes in the cell form of the same general nature are of common occurrence in many species of bacteria, but are not always exactly the same as these, often being much less extreme. Such morphological variants are often very transitory, and can be made to appear and disappear by manipulation of the medium (osmotic pressure, pH, temperature, etc.). However, they sometimes appear in an uncontrollable manner. Some may represent stable mutations, others rapidly progressive reverse mutations, some transitory changes due to environment, like adaptive enzyme formation.

\* *Immunology*, in this sense, is the study of the reaction of infected animals toward infecting microorganisms. It involves study of the effect of substances called *antibodies*, which occur in blood due to infection, upon the infecting microorganisms.

† *Antigens*, as used here, are portions of infecting microorganisms which stimulate the production of antibodies (see footnote above).

**Mucoid Colonies.** The spore-forming organisms just discussed, when cultivated on infusion agar containing a little phenol, produced colonies which were large, viscous and slimy. This kind of colony is common in many species of bacteria, and is called "mucoid" or M. The mucoid material is like an exaggerated capsule or slime layer. It is secreted by the cells in response to various irritating external stimuli (in this case phenol) and, like the slime coating on a garden slug or the mucous secretions of the nasal tract, has protective properties. Mucoid colonies are often called "M" colonies. Like R and S colonies, they not infrequently appear "spontaneously."

**Minute or Small Colonies.** These are *very* minute, often just at the limit of the unaided vision. Cultures derived from minute colonies often have properties (such as virulence) different from cultures from large colonies. A commonly used term for them is dwarf or minute colonies. These colony variants often tend to revert to the larger colony forms. Minute colonies may also be R, S or mucoid.

**H and O Forms** were first described in connection with *Proteus vulgaris*, a non-sporeforming, gram-negative, motile rod commonly found in feces, stagnant water and decaying organic matter. When cultivated on agar plates or any moist, solid medium, *P. vulgaris* usually spreads over the surface quite widely in a thin, smooth, gray, translucent film. The individual cells in such growth are motile, i.e., they have flagella and swim out from the edge of the colony in the film of moisture on the surface of the agar. Such extension of the margin of the colony by motile cells is sometimes called "swarming." This form of growth (it can hardly be called a colony) of the organism has been designated as the *Hauch* or "H" form (the German word *Hauch* meaning a film or veil). It is analogous to the S form of most other organisms.

A second form of growth is observed in which the colonies are small and discrete. In these the *Proteus* bacilli are found to be non-motile, i.e., they possess no flagella. These are called the *ohne Hauch* or "O" form (*Ohne* = German for "without").

Motile and non-motile variants of other organisms are common. Although all motile variants may not have the spreading character (*Hauch* form) of *Proteus*, they are often referred to as H forms, meaning flagellate or motile, while the *ohne Hauch* forms are called O forms because they have no flagella. As will be seen later, the term H is often used to refer to the flagella proteins (H proteins) and the letter O to designate the body or somatic\* substances (O substances) of various bacteria. The meaning of these terms should be clearly understood here. The importance of H and O substances will appear farther on.

**Secondary Colonies.** The formation of small excrescences, papillae or out-growths from ordinary colonies of many species of bacteria, after the first growth is mature and begins to age, is a common phenomenon. The out-growths are called secondary colonies or "daughter" colonies (Fig. 16-6). They may appear on the surface, develop from within, or grow out from the edges. They vary in size, form, numbers, and appearance. The cells in secondary colonies vary from the original in many properties, both morphological and physiological. They represent mutations or adaptations occurring

\* Somatic is from the Greek root *soma*, a body.

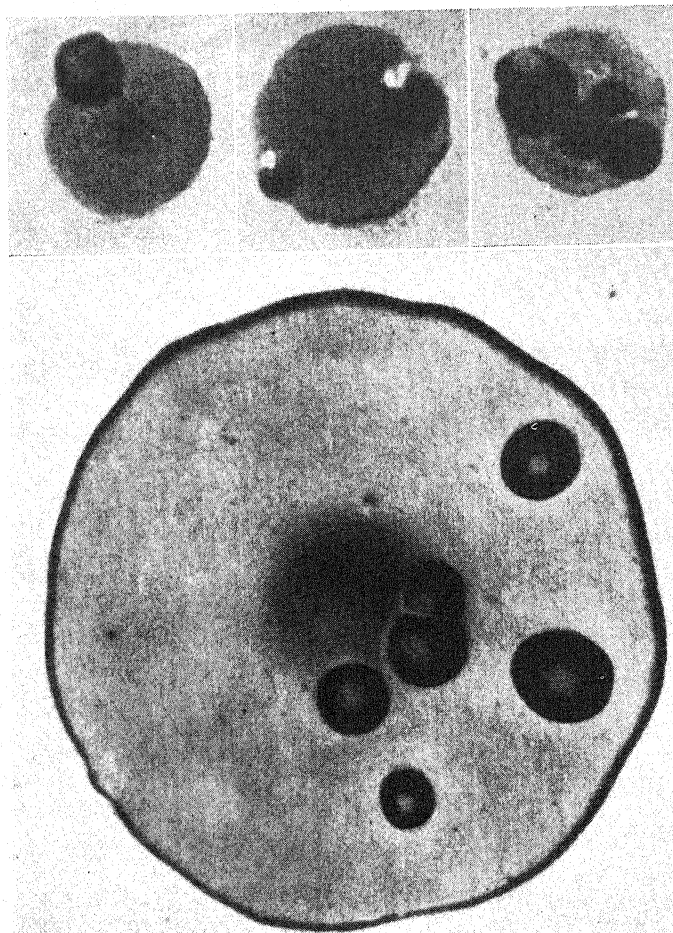


Fig. 16-6. Mutant growths appearing as papillae or secondary colonies of entirely different character from the original colonial growth. (Photos courtesy of Dr. F. J. Ryan, Columbia University, in *J. Bact.*, 1955, vol. 69.)

within the colony during its growth, usually very late, analogous to the non-smooth mutants found in matured broth cultures of *Brucella* due to *d*-alanine formation. They are also analogous to the sector-forming mutants in colonies, described previously.

#### REFERENCES

- Adelberg, E. A., and Myers, J. W.: Modification of the penicillin technique for the selection of auxotrophic bacteria. *J. Bact.*, 1953, 65:348.  
 Braun, W.: *Bacterial Genetics*. W. B. Saunders Co., Philadelphia, 1953.  
 Bryson, V., and Szybalski, W.: Microbial selection. *Science*, 1952, 116:45.  
 Gale, E. F., and Davies, R.: *Adaptation in Microorganisms*. 3rd Symposium, Soc. General Micro., 1953. Cambridge Univ. Press, New York.  
 Lederberg, J., and Lederberg, E. M.: Replica plating and indirect selection of bacterial mutants. *J. Bact.*, 1952, 63:399.  
 Ryan, F. J., Schwartz, M., and Fried, P.: The direct enumeration of spontaneous and induced mutations in bacteria. *J. Bact.*, 1955, 69:552.

## The Systematic Study of Bacteria

IN VIEW of the mutability of bacteria mentioned in preceding chapters, it might be thought that the exact identification and description of a species of bacterium would be an impossible task. This is not so because variation usually occurs within certain, well-known\* limitations. Practically all forms of life vary to some visible extent, some more than others. Creatures like cats or zinnias, with which we are thoroughly familiar, may vary considerably yet we recognize them without difficulty because (a) we are familiar with the genotypes and phenotypes and (b) we recognize certain basic similarities among them.

So with microorganisms; although they may at times vary greatly in one or more respects, they seldom do so in all details at once and, normally, under the uniform conditions of laboratory study, they remain quite recognizable and retain fundamental distinguishing characteristics. It is necessary to know what these are, and how they are usually determined in the laboratory.

### PROCEDURES IN IDENTIFICATION

For purposes of discussion let us proceed as though we have been given an "unknown" organism for complete identification and systematic study.

The first important step in the systematic study of an unknown microorganism is to determine to which of the 8 large groups of microorganisms it belongs and whether other species are present. Let us assume that the organism we are investigating is a bacterium. It is readily visible with an ordinary compound microscope and an oil immersion lens. Its size and shape are within the range of bacterial dimensions and form. It does not require sunlight for growth. It grows readily on simple, organic, non-living culture media without serum. It shows no evidence of yeast-like budding, sex, or animal structure and none of the structures distinctive of molds. It does not produce large bodies or filtrable L forms of the PPLO. From descriptions given earlier in this book there should be no difficulty in identifying an unknown organism as a bacterium and determining that it is not a protozoan, a yeast, a mold, a virus, a rickettsia, an alga or a pleuropneumonia organism (see Tables 1, 2 and 6).

\* Supposedly!

**Purification of Culture.** The next step in an exact study of any bacterium is to separate it from other forms with which it might be mixed. These would introduce error into various biochemical experiments or tests of pathogenicity, pigmentation, etc., performed in the course of the identification. This process is spoken of as *isolation in pure culture*. Many a student\* has fallen into difficulties by assuming the purity of a culture and neglecting this very important step. Microscopic examination of a smear stained by Gram's method may sometimes reveal the presence of contaminants and very often gives a valuable clue as to the genus or family of the organism but cannot be depended upon entirely since many different bacteria look and stain exactly alike. The culture must be purified.

This is done by spreading a drop of the material containing the desired organism on some solid nutrient substance which is known to support growth of a wide variety of organisms. A medium like meat-infusion agar is very useful in such situations. If the unknown will not grow on it, then it may be necessary to prepare a medium especially to resemble as closely as possible the material on which the organism originally grew, if this is known. If not known, autotrophic and heterotrophic media must be furnished with blood, or yeast extract which contains vitamins and growth factors needed by some species of bacteria. If contaminating bacteria are present, they will probably be evident as colonies of distinctive appearance, after incubation of the plates. However, many different bacteria produce colonies very similar in appearance and it may be necessary to examine smears made from selected colonies in order to obtain the desired organism. Even this may yield no useful information, as different species may not only produce colonies closely resembling each other, but possess identical morphological and staining properties as well. In this case we must either decide which of the two kinds of bacteria is the "contaminant" and which the "unknown," or identify both and decide afterward.

For present purposes let us select one of the colonies as the "unknown," and inoculate it on to three plates of blood-meat infusion agar at pH 7.4. Such a medium supports the growth of a wide variety of heterotrophic, saprophytic and parasitic bacteria. It cannot be depended on to support autotrophs. Let us also inoculate three plates of meat- or yeast-extract agar, without blood, in the same manner. If thought necessary, three silica-gel plates may be prepared from some of the solutions noted as serving for the cultivation of strictly autotrophic bacteria such as *Nitrosomonas*. Similar media with organic energy source and agar will serve for such autotroph-like species as *Azotobacter*.

**OBSERVATIONS OF INITIAL GROWTH.** One plate of each kind of medium may now be incubated at 20° C, one at 37° C and one at 55° C. After 24 hours there may be no growth, in which case we may continue incubation for several days. But let us suppose that after 24 hours there is no growth on the plates prepared for autotrophs and near-autotrophs, very sparse or no growth on the extract and infusion plates incubated at 20° C and 55° C, while good growth occurs on plates of both the extract and infusion media held at 37° C. This, then, tells us the approximate optimum temperature for growth and

\* As well as veteran researchers!

also give us an idea as to the kind of media and pH likely to be of use in dealing with our organism. It is clearly aerobic, mesophilic as to temperature and pH, heterotrophic, and probably a saprophyte (though possibly a pathogen) not too fastidious in the matter of organic nutrients.

An inspection of the growth on the agar gives an idea as to the size, shape, color and consistency of colonies. Let us say that the colonies are about 1 to 2 mm in diameter, glistening, convex, circular, opaque, butyrous (butter-like) in consistency and lemon yellow in color.\* A convenient way of recording these facts is to use one of the charts published by the Society of American Bacteriologists.

By carefully transferring a portion of one of these colonies, with a sterile needle into a tube of extract broth, we provide ourselves with a pure culture, which may be studied further as described below.

If no growth occurred on any of the plates inoculated with the original material, we may assume that:

- (a) No living bacteria were present in the inoculum; or
- (b) The temperatures used were not suitable; or
- (c) Some other medium, possibly with a different pH, is necessary; or
- (d) The bacteria may have been strict anaerobes.

Suitable adjustments of conditions must then be made until growth is obtained.

Let us assume that good growth was obtained on the plates and that a pure culture was obtained. The next step in identification is a study of motility, arrangement, morphology, and staining reaction. The first three characters may be determined by observation in a hanging-drop preparation of the growth in broth. Let us assume that our organism is a coccus, although this must be confirmed by microscopical examination of a stained preparation.

**Arrangement and Motility.** A hanging drop prepared with a *young broth culture* is a useful means of determining how bacteria are arranged and their motility. Chains of cocci or bacilli are readily observed, while cubical packets of *Sarcina* can be seen turning over and over like little bundles floating in the medium. *Micrococcus* groups appear to be very irregular, like bunches of grapes but may often appear in pairs. Motility, if present, is easily seen in hanging drops. Let us assume that our unknown is non-motile and a *Micrococcus*.

**Staining Reaction and Morphology.** The next step is to confirm the morphology and observe the staining reaction. Gram's stain is of great value, but one must bear in mind that the method requires experience and that the differentiations it gives are not absolute. Some organisms are gram-positive only when young or when cultivated on blood or serum media. Others are variable under nearly all conditions. Many lose gram-positiveness with aging, or in acid (fermented) cultures. Let us assume that the organism under discussion is gram-positive.

Other morphological features may be looked for at this point. If the organism is rod-shaped it may produce spores; especially if it is gram-positive. No truly gram-negative species of bacteria forms spores. This is best determined by staining an old (at least one week) agar-slant culture with methylene

\* *Potato Medium.* Pigment is often beautifully shown on cubes of potato sterilized with a few drops of water in the bottom of a tube.

blue or by a method of spore staining. A surer test is to emulsify some of the growth in 2 ml of sterile water and heat it at 85° C for ten minutes, afterward inoculating some of the heated material into broth and incubating for several days. If growth occurs, it is practically certain that spores were present since no known vegetative cells withstand 85° C for 10 minutes.

Capsules may or may not be visible, depending largely on the culture medium and whether we are dealing with an R or S form. Sometimes capsules are seen only on organisms in pathological material. They may be determined by capsule stains or darkfield methods. Let us say our organism shows no demonstrable capsules.

The morphology of any organism always varies to some extent. A given variety of bacteria may, in any one culture, produce cells of varying size just as, in a group of men or horses, some may be large and some small. If the bacteria are cocci, some of the individual cells may not be perfectly round and some may be oval. If bacilli, some may be long and thin, others short, oval and thick; some may occur singly, others in pairs or chains or long filaments. But the predominating form, size and arrangement of the cells in a pure culture are usually quite apparent especially when seen growing in different kinds of media, both fluid and solid. Form, size and arrangement of the individual cells are important and reliable differential characters.

Summarizing our knowledge at this point, we may state that we are dealing with a non-motile, gram-positive, unencapsulated coccus, growing well aerobically in irregular clusters on plain, extract medium as well as on blood-infusion medium, at a pH of about 7.4, producing opaque, glistening, lemon-yellow colonies and preferring a temperature of about 37° C. There is still, however, a good deal to learn about our unknown before identification is complete.

**Biochemical Tests.** The experienced bacteriologist would know at once exactly what peculiarities to look for in dealing with a culture of gram-positive cocci which have the characters we have enumerated. For the present, however, we shall put ourselves in the place of a person to whom a gram-positive coccus is a hitherto unknown bacterium.

Since certain enzymes are highly characteristic of certain genera and species of bacteria, the power of our unknown organism to ferment or hydrolyze some of the carbohydrates such as glucose (dextrose), sucrose (saccharose or cane sugar), and lactose (milk sugar), should be tested; also its power to utilize, as hydrogen acceptors, such substances as sodium nitrate and sodium nitrite. Its power to hydrolyze gelatin, coagulated serum, the casein in milk, starch, fat, etc., should also be investigated. All of these are simple procedures.

In any of these tests it is important that the medium and conditions of incubation support *good vigorous* growth of the organism. For example, for certain bacteria to produce gas by the decomposition of lactose, it has been shown that from 40 to 390 million cells of this species must be present in each ml of the test medium. A negative result in the absence of normal, good growth is obviously of no value. In order that uniform, standard, and authoritative results may be obtained, it is recommended that recognized, documented procedures such as those outlined in the "Manual of Bacteriological Methods," published under the auspices of the Society of American Bacteriologists be used throughout. It should be emphasized that results obtained

in empirical media of the complex organic type, like extract or infusion broth, can vary with changes in the quantity and quality of such ingredients as peptones, yeast extracts and meat extracts; presence or absence of certain kinds of hydrogen donors and acceptors; pH; temperature; and other factors. These variables are always standardized as far as possible and are carefully recorded for comparative purposes by competent workers.

**FERMENTATION TESTS.** When dealing with an unknown, each tube of broth should contain a small inverted vial (placed there *before* sterilization) (Fig. 17-1) to catch any gas that may be formed. Gas might otherwise pass off into the atmosphere and not be detected. Two organisms, both of which ferment the same carbohydrate, may be sharply differentiated if one forms gas while the other does not.

When gas is produced from carbohydrates by bacteria, it is *prima facie* evidence of fermentation and is always accompanied by souring or acid formation since the gas is derived from the formic acid resulting from fermentation. However, fermentation often occurs without gas production, and then acid formation is our only evidence that the organism has metabolized the carbohydrate. Sometimes only alkaline substances are produced from carbohydrates.

Acid or alkali may be detected by adding to the medium an indicator or dye, such as brom-cresol purple which changes from purple to yellow in the presence of acid. Phenol red turns from yellow to red in the presence of alkali. The change in color of the indicator is our proof of fermentation or metabolic use of the carbohydrate. However, some species can metabolize the acids produced by fermentation. Others may produce ammonia from amino acids in sufficient quantity to mask acid production. In other instances, if too much buffer is used in the medium it, too, will mask acid or alkali production. These possibilities can be allowed for, but must be kept in mind.

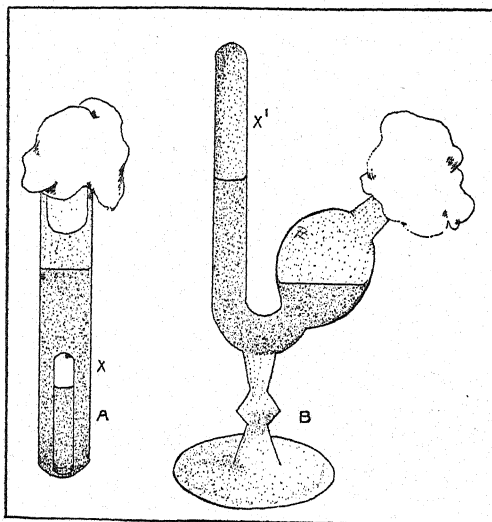


Fig. 17-1. Types of apparatus used for collecting gas produced by bacteria. A, Durham tube (gas has collected at X). B, Smith tube (gas has collected at X').



Observations of growth should be made every twenty-four hours in order that the culture may not revert to an alkaline reaction before acid formation has been noted. Variant strains of the same species may differ considerably in the *rate* at which these reactions are brought about and ample time for observation must therefore be allowed.

**GASES PRODUCED BY BACTERIA.** The nature of the gas formed by bacteria may be of differential value. Two gases commonly given off by bacteria during fermentation of carbohydrates, etc., are carbon dioxide and hydrogen in various proportions. It is often of importance to determine the ratio of carbon dioxide to hydrogen. The differentiation is easily made by adding strong NaOH solution to the fermentation tube. This absorbs the CO<sub>2</sub>, leaving H. The difference in level of fluid in the fermentation vial is easily estimated if the level was marked before adding NaOH.

Many organisms, in their metabolism of proteins or protein-digestion products, e.g., cystine, taurine and other sulfur compounds, set free *hydrogen sulfide*, often in large amounts. This gas is one of the most noticeable in connection with putrefactive processes. Some organisms may be identified or differentiated from others by their power to produce H<sub>2</sub>S. Thus, *Salmonella schottmülleri*,\* a cause of gastroenteritis, produces hydrogen sulfide in its decomposition of amino acids, while *Salmonella paratyphi*,† a similar species, does not. The test therefore has differential or diagnostic value.

*Methane* is another gaseous product of bacterial metabolism. It results usually from the metabolism of carbohydrates and other organic compounds under anaerobic conditions. Carbon is a good hydrogen acceptor and is readily reduced. In swampy places anaerobic bacteria attack the carbohydrates derived from dead vegetation and sometimes large amounts of the gas are given off. The bubbles seen arising during the summer time in woodland swamps are nearly always largely methane. Microorganisms in sewage digestion tanks at sewage disposal plants produce such large quantities of methane that it is economically feasible to collect it in tanks and use it as fuel in furnaces, etc.

Some organisms may produce poisonous gases under certain circumstances. For example, *Pseudomonas aeruginosa* is said to produce hydrogen cyanide (HCN), while a number of species of *Corynebacterium*, especially *C. diphtheriae*, produce hydrogen telluride when cultivated, as is frequently done, upon medium containing potassium tellurite. Many bacteria, especially saprophytic species, also produce large amounts of ammonia and nitrogen. These result from decomposition of proteins and other nitrogenous compounds.

**PROTEOLYSIS.** In addition to the above tests, tubes containing common proteins may be inoculated to determine the ability of our unknown to attack different proteins. These may be incubated with the carbohydrate tubes.

**GELATIN.** A tube of solidified nutrient gelatin‡ is inoculated by "stabbing" a wire, having the desired bacteria upon it, down into the depths. Some workers prefer to incubate the gelatin at 20° C rather than at body temperature. At the lower temperature the gelatin remains solid except where di-

\* Now *S. paratyphi B*. (See footnote on page 244.)

† Now *S. paratyphi A*. (See footnote on page 244.)

‡ Ordinary extract or infusion broth containing 10 per cent gelatin.

gested by the organism, and the shape of the portion liquefied may be observed. This was formerly regarded as of great significance, but is really of much less value than other tests. The time lost in waiting for growth to occur at 20° C (if indeed it occurs at all) is much more valuable than the information as to the form of the liquefied area. Gelatin cultures held at 37° C liquefy completely due to the temperature but may be placed daily in the refrigerator for a sufficient time to allow them to solidify if undigested. This should be continued for two weeks unless evidence of digestion is obtained sooner. An uninoculated tube of gelatin is incubated with the others to serve as a guide in refrigeration time. Those tubes in which the gelatin fails to solidify may be marked "+" or "digested." *The tubes should not be shaken* while warm, as growth, and a small amount of gelatin digestion, frequently occurs *only in the surface layer* and this would be masked were it mixed with the bulk of the warm, fluid culture.

**SERUM DIGESTION.** Coagulated serum may be prepared by mixing three parts of horse or beef serum with one part of nutrient broth. Glucose is sometimes incorporated in a concentration of 0.25 per cent. The serum is coagulated and sterilized at the same time. The slants are inoculated when cool, by smearing a loopful of broth culture or growth from agar over their surfaces.

When attacked by bacteria the serum usually becomes brownish and translucent and the growth appears to sink inward. Total liquefaction sometimes occurs in forty-eight hours at 37° C, especially in cultures of aerobic sporeforming bacilli, but is often delayed for as long as two weeks.

**ACTION ON MILK.** Milk has a pH of around 6.8 when fresh. It is an ideal culture medium for many bacteria.

For use as a bacteriological culture medium, skimmed milk is tubed in 5-ml amounts and sterilized by autoclaving (steam pressure cooker: 120° C for 10 minutes). If an indicator, such as litmus or bromocresol purple, be added, fermentation of the lactose may be detected. Rennet production may be inferred if the milk is curdled (provided this is not due to souring; a point difficult to determine if fermentation of the lactose also occurs). Hydrolysis of the casein often follows coagulation. The milk then becomes brownish and translucent and the clot disappears.

Another method of testing the ability of many organisms to hydrolyze test substances like serum fat, or starch is to mix the test substance with nutrient agar, pour into Petri plates and, when solid, heavily inoculate the surface in streaks or spots. After good growth has occurred a reagent, reacting with the test substance to produce some distinctive appearance, is flooded over the surface of the agar. If hydrolytic enzymes have been produced the colonies will be surrounded by zones where no reaction (or a distinctive reaction) occurs. For example, gelatin or serum may be added to the agar. By flooding the plate (after incubation) with several ml of a solution consisting of H<sub>2</sub>O, 100 ml; HgCl<sub>2</sub>, 15 gm; HCl, 20 ml, unhydrolyzed gelatin or serum will be coagulated to a white opaque appearance. The colonies of hydrolyzing organisms will be surrounded by a clear zone. Starch plates may be made similarly, and treated with Lugol's iodine solution. The starch-hydrolyzing colonies will be surrounded by colorless zones. The remainder of the plate will turn dark blue.

**DETERMINATION OF LIPOLYSIS.** Many organisms produce enzymes capable of hydrolyzing one or more fats or oils (lipids). Methods for detecting lipolysis have not been widely developed or used. One of the inconveniences in studying lipolysis is difficulty in bringing the lipid substrate into intimate contact with the organism. This contact is desirable since lipolytic enzymes often do not diffuse well into culture media. Another difficulty is in making the hydrolytic effect evident.

In a method eliminating some of the difficulties a plate of nutrient agar is streaked with the organism to be tested. The oil (or melted fat) is then applied to the surface of the plate in a fine spray. After incubation, examination of the lipid droplets is made with a low power microscope. Those that have been hydrolyzed are readily detected by their opaque appearance. This and another method are seen in Figure 17-2.

**BACTERIAL REDUCTIONS.** A physiological property characteristic of many bacteria is the power to reduce various compounds.

**NITRATE REDUCTION.** Incubate the bacteria being investigated in broth containing about 0.1 per cent of sodium *nitrate* ( $\text{NaNO}_3$ ). After forty-eight hours and at other intervals, a test is made for the presence of *nitrites* ( $\text{NaNO}_2$ ) by withdrawing a little of the culture from the *bottom* of the tube and immediately adding to it a drop of sulfanilic acid solution\* and a drop of dimethyl-alpha-naphthylamine solution.† Or allow the drops to settle to the bottom of the whole culture. It is better to test the *bottom layers* of the culture rather than mixing the whole culture, because slight reduction sometimes occurs at the bottom of the tube and nowhere else, due to lower oxygen tension in the depths of the tube. The presence of oxygen interferes with nitrate reduction. Oxygen is present in greater concentrations in the upper layers of fluid.

The development of a red or brown color denotes the presence of *nitrites*, but the failure of this color to develop raises a question. Either (a) the nitrate has not been attacked, so that no nitrite is present or (b) nitrite has been formed but also attacked and reduced to free nitrogen or ammonia. A test to see whether any nitrate remains may be made by adding a little pulverized zinc. This reduces any remaining nitrate to nitrite (which may be tested for as above) and tells us whether or not the organism has reduced *all* of the nitrate. If the test with Zn is positive (i.e., nitrate is still present), the original test for nitrites having been negative, then it is clear that the organism did not attack the nitrate at all. If both the nitrate and nitrite tests are negative then it is obvious that the organism reduced all the nitrate as well as nitrite to nitrogen or ammonia.

**NITRITE REDUCTION.** The latter point, i.e., ability of the organism to attack nitrite, may be determined separately by testing the ability of the organism to

\* Sulfanilic acid solution

Glacial acetic acid.....	100.0 ml
Water.....	250.0 ml
Sulfanilic acid.....	2.8 gm

† Dimethyl-alpha-naphthylamine solution

Glacial acetic acid.....	100.0 ml
Water.....	250.0 ml
Dimethyl-alpha-naphthylamine.....	2.1 ml

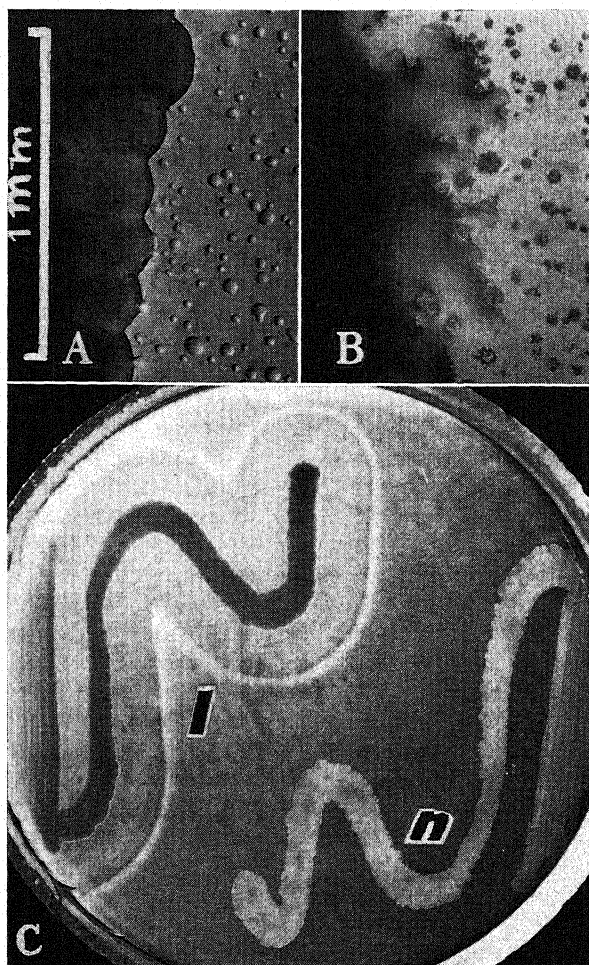


Fig. 17-2. Lipase production and its detection. At *A* is seen the margin of a colony of *Micrococcus* sp., on a plate of agar sprayed with a fine mist of olive oil. No lipase is evident since all of the droplets of oil have remained unchanged, even in contact with the colony. At *B* is seen the irregular margin of a colony of *Serratia*\* on agar sprayed with oil. Lipase activity has caused the oil droplets to become deformed and optically dense and refractive near, as well as at some distance from, the colony. At *C* is shown a plate containing agar in which fat is emulsified and to which has been added a small amount of Nile blue sulfate (a dye which turns blue in the presence of lipolysis). On this plate are seen a strain of *Micrococcus* producing lipolysis (zone of color change and emulsion destruction, *l*), and a strain not producing lipolysis (no zone of color change or emulsion destruction, *n*). (*A*, courtesy of Dr. C. J. E. A. Bulder, in A. van Leeuwenhoek J. Micr. and Serol., 1955, vol. 21; *B*, same author, unpublished; *C*, courtesy Dr. M. E. Davies, in J. Gen. Micr., 1954, vol. 11.)

\* A genus of red-pigmented Enterobacteriaceae.

destroy the nitrite in cultures known to contain it. This is done by incubating cultures containing quantities of nitrite so small (about 0.002 per cent) as just barely to give a positive test for nitrites. If the organism is capable of reducing nitrite, the culture will soon lose its power of reacting positively to the nitrite reagent, because the organism will quickly reduce all of such a small amount of nitrite. A sterile control tube should be tested at the same time, since illuminating-gas fumes, as from Bunsen burners, often contain nitrous acid which may be absorbed by the medium and give a slight reaction.

**REDUCTION OF LITMUS.** The ability of an organism to reduce other substances than nitrates and nitrites is often investigated. Litmus, for example, is often used as an acid-indicator in milk cultures, but it also serves to show whether or not the organism has strong reducing powers by becoming entirely decolorized when reduced. Just enough is added to the medium before sterilization to give a definite color.

**THE "REDUCTASE" TEST.** Methylene blue is similarly decolorized by many organisms. The dye is used as a hydrogen acceptor in respiration (see section on bacterial respiration). Many other compounds are similarly utilized. Standardized solutions of methylene blue are often added to market milk samples to estimate, roughly, whether a few, a moderate number, or enormous numbers of bacteria are present. When great numbers are present, the blue color may disappear almost immediately. Several other oxidation-reduction dyes are now used besides methylene blue: tetrazolium salts, neutral red, resazurin, etc.

**INDOLE.** Indole is derived from the amino acid tryptophan as a result of hydrolysis by certain species of bacteria. Most peptones contain tryptophan but if a medium is used in which it is not *known* to be present it must be added if one is to test for indole production. Cultures are incubated for forty-eight to seventy-two hours. Indole reacts with acidified solution of Ehrlich's reagent (para-dimethyl-amido-benzaldehyde)\* to produce a pink compound.

The culture to be tested is first shaken with 1 ml of xylol. Indole is soluble in xylol and is concentrated in it and carried to the surface by the solvent after a minute of standing. Six drops of Ehrlich's reagent are then *gently* added to the culture and are made to remain in a layer between the xylol and the medium. If indole is present a pink color forms in a few minutes as a "ring" at the junction of the xylol and the reagent. Pink compounds *not* soluble in xylol sometimes develop in the presence of Ehrlich's reagent if the xylol is not added first. These are not due to indole.

There are many other tests which are used in microbiology; some only for very specific purposes in diagnostic work; others in special phases of research. A few are described in other parts of this book, and others in the literature.

**Rapid Microtechniques.** By certain modifications of technique, many of the tests described above may be made more economically and also greatly speeded up. Three general types of rapid microtechnique are available. First, since bacteria rapidly multiplying in the logarithmic phase are most active in all enzyme functions, one may greatly speed up their effects by adding heavy

\* Ehrlich's reagent

Ethyl alcohol (95%).....	380 ml
HCl (conc.).....	80 ml
Para-dimethyl-amido-benzaldehyde.....	4 gm

suspensions of young, actively-growing organisms from an agar-slant culture to small amounts of the test medium (previously warmed to the temperature desired and held in a water-bath during the period of incubation). In this way, instead of incubating cool, sparsely inoculated test cultures for one or more days slowly to develop populations sufficiently large to induce the desired change, the ready-made population consisting of billions of young, active organisms in the heavy suspension from the agar slant set to work immediately and bring about the desired characteristic changes within a few minutes or hours. By using 1.0 ml amounts of test fluids in small tubes and adding a few drops of very heavy bacterial suspensions, a great many tests may be done economically in a small space and short time.

Second, one may centrifuge 10 ml of a young broth culture, remove the supernatant broth with its various unknown ingredients, and resuspend the bacteria in a few drops of saline solution or distilled water. For a fermentation test, a drop of this heavy suspension of young, active cells is placed in a small (5 x 50 mm) tube with 0.5 ml of acid-alkali indicator and a drop of the desired sugar solution. The results may be read in 6 to 12 hours. Modifications of this procedure permit tests to be made quickly for urease production, nitrate reduction, gelatine hydrolysis, etc.

A third type of procedure involves inoculation of 15 ml of melted, nutrient agar at 40° C, in a tube, with a heavy suspension of young, active, cells. The agar is then poured into a Petri dish and allowed to solidify. One then places on the surface small, paper disks, previously dried after saturation with solutions of test carbohydrates and suitable indicators. The test substance diffuses into the agar around the paper disk and is acted upon by the bacteria in the agar.

**Identification of the Unknown Organism.** After completion of the tests as described above, a tabulation is made of the results. For the organism under investigation, let us assume that they are as follows:

Pigment (as observed in colonies on agar)—lemon yellow.

Lactose—fermented; no gas.

Glucose—fermented; no gas.

Sucrose—fermented; no gas.

Salicin—not fermented.

Gelatin—slowly liquefied.

Agar slant and cubes of potato—good growth, soft, moist and glistening, lemon yellow in color.

Broth—turbid, sediment is yellow; faint pellicle (scum).

Milk—coagulated, acidified.

Nitrates—reduced to nitrites.

Indole—not produced.

How are we now to determine the genus and species of the cocci which we have been studying?

**Need for Keys.** Although every experienced bacteriologist has at his finger tips, so to speak, all of the distinguishing cultural reactions and other identifying characters of the organisms with which he is working, it is unusual, to say the least, to find one who knows *all* the characters of *all* the species.

When an unknown organism is encountered which must be identified, the main morphological and tinctorial features are determined in some such manner as just described and then recourse is had to *keys* or other reference works. Probably the most useful key for general bacteriological use in the United States is "Bergey's Manual of Determinative Bacteriology."\*

**USE OF KEYS.** In order to use "Bergey's Manual" we should first determine to which of the five orders of the class Schizomycetes our culture belongs. On pages 65 and 66 of the 1948 edition of the manual is to be found a brief synopsis of the characters used to differentiate the five orders and suborders. Obviously the species in question is neither spiral, elongate or flexuous, filamentous, sheathed nor characterized by iron or sulfur deposits (Orders III, IV, and V). Neither is it branching (Order II). It does not possess photosynthetic pigment nor form long stalks, hence does not belong in suborders II or III of Order I. We are thus left to consider Suborder I, the Eubacteriineae.

On pages 67 and 68, we find a synoptic description of the families of the Suborder Eubacteriineae. Our organism is spherical and may therefore fall into either family I (Nitrobacteriaceae), family V (Micrococcaceae), family VI (Neisseriaceae) or family VII (Lactobacteriaceae). It is obviously not a member of the other families, which include only rod-shaped or filamentous bacteria. To settle the choice between families I, V, VI, and VII, more exact information is necessary and we turn to page 69. On page 69 *et seq.*, we find that in only one genus of family I, the *Nitrosococcus*, are the organisms spherical. But the organisms of this genus do not grow on ordinary heterotrophic culture media, whereas our "unknown" does, and will not grow autotrophically. This, therefore, leaves us to consider families V, VI, and VII and to them we turn our attention.

Reference to page 295 makes it clear that we are not dealing with Neisseriaceae (family VI) since they are gram-negative; and we eliminate at present also the Lactobacteriaceae (page 305, tribe Streptococceae) since our organisms grow well on ordinary extract agar without blood and do not occur predominantly in chains but in irregular clumps and masses as well as in pairs.

Family V (Micrococcaceae), therefore, would seem to be our objective, and we are referred to page 235. Here we find that the descriptions of genera II and III (*Gaffkya* and *Sarcina*) do not correspond with the organism in question, and that we must search in genus I. The organisms of this genus resemble each other so closely that it is necessary to consider carefully the characters of the individual species.

A short study of the data we have already obtained by our cultural tests shows that our organism corresponds closely with the description of *Micrococcus citreus* (page 242), since it produces a lemon-yellow pigment, liquefies gelatin, ferments lactose, grows on potato with lemon-yellow pigment, reduces nitrates to nitrites and fails to produce indole.

A further check upon the identity of the culture may be made by testing other fermentable substances and comparing various characters of organisms closely resembling it, such as *Micrococcus flavus*. A few repetitions of the

\* All names of bacteria given in this book are based on the 1948 edition of "Bergey's Manual" unless otherwise noted except in a few instances where official groups have more recently adopted superseding names.





Fig. 17-3. Injection of a chick to test the toxigenicity of diphtheria bacilli. The injection is intraperitoneal (abdominal cavity). The material is 4 ml of 48-hour broth culture of the organism to be tested. Guinea pigs, rabbits, and other animals are often used. (Photo courtesy of the U. S. Public Health Service, Communicable Disease Center, Atlanta, Georgia.)

tests usually serve to confirm the diagnosis, or prove it to be in error, necessitating further study.

Pathogenicity may be determined by injecting 0.5 ml of a twenty-four-hour broth culture intravenously and subcutaneously into a rabbit, guinea pig or other animal (Fig. 17-3). If our unknown is truly pathogenic, abscesses will probably form or the animal may die. Some strains are more pathogenic than others. Further details concerning micrococci will be given when these genera are taken up specifically later.

#### REFERENCES

- Adelberg, E. A.: The use of metabolically blocked organisms for the analysis of biosynthetic pathways. *Bact. Rev.*, 1953, 17:253.
- Bergey, et al.: *Manual of Determinative Bacteriology*. 6th ed. Williams & Wilkins Co., Baltimore, 1948.
- Bulder, C. J. E. A.: Some observations on the lipolytic activity of microorganisms and a new method for its detection. *Antonie van Leeuwenhoek J. of Micr. and Serol.*, 1955, 21:28.
- Conn, H. J., Editor: *Manual of Bacteriological Methods*. Biotech Publications, Geneva, N. Y., and/or Soc. of Amer. Bacteriologists, c/o Williams & Wilkins Co., Baltimore, Md.
- Cowan, S. T.: Rapid micromethods for bacteriology. *Lab. Prac.*, London, 1953, 2:241.
- Greene, R. A., and Larks, G. G.: A quick method for the detection of gelatin liquifying bacteria. *J. Bact.*, 1955, 69:224.



- Ordal, E. J., and Earp, B. J.: Cultivation and transmission of etiological agent of kidney disease of salmonid fishes. *Proc. Soc. Exp. Biol. and Med.*, 1956, 92:85.
- Schaub, T. G., and Foley, M. K.: *Methods for Diagnostic Bacteriology*. 4th ed. C. V. Mosby Co., St. Louis, 1952.
- Smith, N. R., Gordon, R., and Clark, F. C.: *Aerobic Sporeforming Bacteria*. Agriculture Monogr. No. 16, U. S. Dept. of Agric., Washington, D. C., 1952.
- Underwood, W. B., and Perkins, J. J.: *Textbook of Sterilization*. 3rd ed. Charles C Thomas, Springfield, Ill., 1955.
- Verhoeven, W.: Studies on true dissimilatory nitrate reduction. V. A. Van Leeuwenhoek J. Micr. and Serol., 1956, 22:385.
- Weaver, R. H., et al.: Rapid micro-technics for identification of cultures. A series of papers. *Am. J. Clin. Path.*, 1951, 27:195; *J. Bact.*, 1947, 54:28; *J. Lab. and Clin. Med.*, 1948, 33:1338.

## Destruction, Removal and Inhibition of Microorganisms

### 1. BASIC PRINCIPLES

AN IMPORTANT phase of microbiology is knowledge of methods for killing, inhibiting (preventing growth of), and removing microorganisms. As species of microorganisms vary in the ease with which they may be destroyed, inhibited or removed, and as the situations in which they may occur differ greatly (e.g., blood, foods, water, sewage, soil, milk, bedding, etc.), no one or two methods are generally applicable. Each situation is a problem in itself, and the methods employed must depend on the knowledge, ingenuity and skill of the operator. There are basic facts, however, which guide the procedure in any given situation.

There are four main reasons for killing, inhibiting or removing microorganisms. They are: (1) to prevent infection of men, animals and plants; (2) to prevent spoilage of food and other commodities; (3) to prevent interference by microorganisms in various industrial processes depending on pure cultures; (4) to prevent contamination of materials used in pure-culture work in laboratories (diagnosis, research, industry, etc.), so that studies of the growth of one kind of organism in a particular medium or infected animal may not be confused by the presence and growth of others, at the same time.

#### DEFINITION OF TERMS

Several new, special terms are used in this chapter. These may be explained as follows:

**Sterilization.** Sterilization means the freeing of any object or substance from all life of any kind. This is accomplished usually by heat but sometimes, in special cases, by the use of chemicals, ultraviolet irradiation or filtration.

**Bactericide.** Any substance or agent killing bacteria is a bactericide or bactericidal agent. The suffix, *-cide*, indicates "killer" and is used with germ, virus, etc.

**Disinfection.** This means the killing or removal of organisms capable of

causing infection and does not necessitate sterilization. However, some processes of disinfection accomplish sterilization. Disinfection is usually accomplished by chemicals like carbolic acid (phenol), formaldehyde, chlorine, iodine or bichloride of mercury. In the case of milk, disinfection, *but not sterilization*, is brought about by *pasteurization*, a heating process to be described.

A **DISINFECTANT** is an agent accomplishing disinfection. The term is often used synonymously with *antiseptic*. One ordinarily thinks of disinfection and disinfectants as applicable mainly to situations and objects not part of the body: floors, dishes, laundry, bedding, etc. However, one often hears of "disinfecting a wound."

**Sepsis** is the growth of harmful microorganisms in living tissue.

**Asepsis.** In a strict sense, asepsis is the absence of infectious microorganisms from living tissue, but the term is usually applied to any technique designed to keep *all unwanted* microorganisms out of any field of work or observation. The work of a microbiologist and of a surgeon involves aseptic technique. The surgeon and his assistants have sterile instruments, handle them with sterilized gloves, cover the patient with sterilized sheets except for such area as is necessarily uncovered, and wear sterilized caps, gowns and masks to prevent infected dust, droplets of saliva, perspiration or sputum from entering the sterile field and possibly infecting the patient. The patient's skin cannot be absolutely *sterilized* without injury, but the site of the operation is *disinfected* as thoroughly as possible by applications of some suitable disinfectant.

In microbiology the worker uses sterilized culture media and sterilized glassware kept sterile, until the moment of use, by coverings of paper and cotton plugs and by aseptic technique; i.e., avoidance of touching sterile materials with hands or unsterile objects, exclusion of dust, etc.

**Antiseptic** is an ill-defined term, closely allied to "disinfectant." A disinfectant is often used as an antiseptic and vice versa. Antiseptics are substances which kill or inhibit microorganisms, especially in contact with the body.

**Bacteriostasis; Bacteriostatic Agents.** These are substances or conditions which do not immediately kill the bacterial cell but which inhibit multiplication so that the microorganisms die only after hours, days or years without significant increase in number. Important bacteriostatic agents are desiccation, very low temperatures, antibiotics (Terramycin, penicillin, Aureomycin, etc.), sulfonamide drugs, and certain dyes like crystal violet. Others will be discussed later.

The distinction between a disinfectant and a bacteriostatic agent is interesting but entirely arbitrary and depends on what one may call killing "quickly," and what one may mean by inhibiting or, what amounts to the same thing, killing "slowly." One could say that death within a time less than the maximal for one generation (under optimal growth conditions) might be regarded as more than mere prevention of multiplication and, therefore, "true killing" or "disinfectant" action; while, if death were delayed beyond such time, one might regard death as due only to factors other than the agent applied, acting on cells which have merely been prevented from multiplying. This could represent "true bacteriostasis." There are many obvious difficulties in making such a distinction. The term bacteriostasis is, however, widely used and generally means delayed death, i.e., killing only after some hours, days, or longer, rather than in 10 to 30 minutes.

## PRINCIPAL METHODS

We may consider four general classes of means of killing and/or inhibiting, as follows:

1. **Destruction of Physical Structure.** There are many substances which act upon colloidal suspensions (of which protoplasm largely consists) to cause the suspended particles (*discontinuous phase*) to change their relationship to each other and to the suspending fluid (*continuous phase*). The mass becomes solid like hard-boiled egg or like "clotted" (soured) milk. *Coagulation* is said to have occurred. In the case of the egg, heat coagulation occurred; in the milk, acid or chemical coagulation.

Colloidal particles may be thrown out of suspension if their negative charges are neutralized; for example, by certain metallic cations: hydrogen ions ( $H^+$ ), copper ions ( $Cu^{++}$ ), zinc ions ( $Zn^{++}$ ), iron ions ( $Fe^{+++}$ ), etc. Several heavy metals are in common use as disinfectants or bactericides, e.g.,  $CuSO_4$ ,  $AgNO_3$ ,  $HgCl_2$ ,  $ZnO$ . Of metallic ions it may be said that, in general, coagulating power is exponentially related to valency and atomic weight; the trivalent, heavy metals having much greater than one third more coagulative power than bivalent metals.

Certain organic substances are also important as coagulants; among them, alcohol, phenol, formaldehyde, and related and derived substances.

All of these reactions, organic and inorganic, are included in the term *chemical coagulation*.

The general rule may be stated that any agent inducing coagulation, or any change like it, is lethal to living cells.

2. **Non-specific Chemical Combinations.** Various chemically active substances will combine indiscriminately with any and all proteins and protoplasm. Chlorine is such a substance; iodine another. Creosotes, carbolic acid (phenol), and formaldehyde are others. Lye (strong alkali) and strong acids are destructive of nearly all organic matter. We will discuss details of the practical uses of such substances later. Such substances are entirely non-specific in their action. That is, they will combine as readily with body tissues, or with casein, feces, mucus, blood, wood or leather, as with bacterial protoplasm.

3. **Specific Chemical Combinations and Bacteriostatic Agents.** There is a group of substances which, in relatively low concentrations, can enter certain cells and interfere with, or completely stop, the action of one or more *specific molecular groups* in a *particular enzyme* or enzymes in such cells. Substances of this nature are represented by sulfonamide drugs and antibiotics which are discussed more fully farther on.

4. **Non-specific Bacteriostatic Methods.** There are several methods of stopping (or very greatly slowing) cell function which are entirely physical and affect the cell as a whole. All are fundamentally methods of depriving the cell of liquid water: (1) drying; (2) immobilizing the water by changing it into solid ice; (3) drawing most of the water out of the cell by immersing it in a fluid of high osmotic pressure such as pickling brines or preserving syrups. These methods were discussed in Chapter 12.

The action of bacteriostatic agents is characteristically reversible; that is, the organisms can be reactivated if the bacteriostasis has not been too pro-

longed. This differs for different agents. For example, organisms treated with bichloride of mercury can be reactivated by treating them with  $H_2S$ , up to an hour or so following treatment with  $HgCl_2$ . The  $H_2S$  precipitates the Hg as  $HgS$ . Many species of bacteria held dormant by desiccation in vacuo can be reactivated after 25 years or longer.

**Combined and Variable Effects.** While we have cited several distinct kinds of deleterious actions on living matter: (a) coagulation, (b) non-specific chemical combinations; (c) specific chemical combinations; and (d) dehydration, actually many widely used and effective disinfectant substances and chemotherapeutic drugs act by more than one of these mechanisms. In fact, many microbicidal and/or microbistatic actions are, at best, only partly understood.

#### THE ROLE OF HYDRATION IN DISINFECTION

The role of *hydration* in coagulation is clearly shown by experiments on the effect of heat on egg albumen under various degrees of hydration. The results of some of these experiments are shown in Table 9.

Table 9. *Effect of Hydration and Heat on Egg Albumen.\**

WATER CONTENT (Per Cent)	APPROX. COAGULATION TEMPERATURE (° C.)
50	56
25	76
15	96†
5	149
0	165‡

\* Albumen is one form of protein, closely resembling protoplasm.

† Boiling water = 100° C.

‡ 165° C. = Oven temperature.

Obviously coagulation proceeds best where protein is well hydrated. The less the degree of hydration, the more resistant is the protein to coagulation. The same principle holds true in coagulation by chemicals.

The resistance of bacterial *endospores* to heat and chemical disinfectants may probably be explained as being due either to the dehydrated condition of their protoplasm or to the fact that the free water of the protoplasm became closely united or *bound*\* when the spore was formed. In either case the water no longer functions as a separate aqueous phase. Examples of the resistance of dehydrated protein to the action of coagulative agents are seen in the difficulty of making dried-egg powder coagulate. Heated in a test tube it will turn brown or char, but it will not coagulate unless a considerable quantity of water has been added. Of course, mixing dry egg with dry chemical disinfectants such as powdered bichloride accomplishes nothing at all till moisture is added. The same is true of dried-milk powder. Chemical reactions necessary to the action of a variety of disinfectants, are facilitated by the presence of water, since *ionization* is essential, as well as hydration of protein.

\* The apparent dryness of a hard-boiled egg is a good illustration of "bound water."

## FACTORS AFFECTING CHEMICAL DISINFECTION

Of basic importance in disinfection with chemicals is contact between the microbicidal agent and the microorganism. In dealing with aqueous solutions of disinfectants, "wetness" (low surface tension) is a determining factor. There are two aspects of this factor: first, the accumulation (adsorption) of surface-tension-reducing disinfectants on the surfaces of cells; second, the effect of surface tension reducents on the wetting and spreading properties of the solution. Both affect *contact* between disinfectant and microorganisms.

**Contact.** Upon adding phenol to a broth culture of bacteria, contact between disinfectant and bacteria is immediate. The bacteria float naked, as it were, and are reached by the disinfectant in effective concentration without delay, partly because phenol *lowers surface tension* and is therefore *adsorbed* upon their surfaces. Once the surface-active substance is in contact with the organism, further action depends on specific toxicity of the anion, cation, pH, temperature, species of organism, whether or not the disinfectant penetrates readily inside the cell, and other factors.

**WETNESS.** Surface tension reducents enhance the effectiveness of disinfectants by increasing their wetness. The reason for this has been given in Chapter 12.

Soap is a good surface tension reductent. Soapy, or saponated, solutions therefore wet surfaces thoroughly. Phenol and related compounds, like cresols, also lower surface tension and are, in and of themselves, powerful germicides besides. A combination of soap, carbolic acid and cresols would, therefore, seem to have exceptional possibilities as a disinfectant. Indeed, mixtures containing these substances in effective proportions are widely used in hospitals, laboratories, etc. Products of this type are available on the market, or can be made up as *Liquor Cresolis Compositus* from the U. S. Pharmacopeia. Solutions of iodine with low surface tension are now available commercially; e.g., *Wescodyne*.

**COMPETITIVE ADSORPTION.** An excess of soap, however, will interfere with the adsorption of disinfectants. The addition of more than minimal amounts of a bactericidally ineffective but very surface-active substance like soap to disinfectant solutions is likely to be disadvantageous. The relatively inert soap is adsorbed on the bacterial surfaces to the exclusion of the disinfectant. Such displacement of one surfactant\* by another is often called *competitive adsorption*.

If organisms are coated with an oily or waxy film, as is the case naturally with tuberculosis bacilli, even when a disinfectant reaches them it affects them with difficulty unless it possesses the special physical property of lowering surface tension, thus allowing it to wet the wax.

There is another aspect of the property of wetness, relating to nutrition. If certain harmless surface-tension reducents are added to *nutrient* solutions, they facilitate contact of the nutrient fluid with the organisms and thus stimulate growth. The use of this principle in cultivating waxy tubercle bacilli and diphtheria bacilli and other organisms is discussed in the sections dealing with those organisms.

\* Term commonly used for any substance active at surfaces; surface-tension reducents in general.

**Synthetic Detergent\* -Disinfectants.** There is a whole class of disinfectants which are very active surface-tension reducents. The first was discovered by Domagk who, in 1936, described the properties of dodecyl-dimethyl-benzyl-ammonium chloride. This is a quaternary ammonium salt, and is representative of a large number of similar compounds which have since been developed commercially as disinfectants and/or detergents.† Many combine microbicidal and soap-like properties.

Essentially, they are ammonium halides in which the hydrogen atoms have been replaced by organic radicles. A commercially familiar example is *Zephiran*. (See Fig. 18-1.)

**Chemical Structure and Activity.** In general, the more effective of these compounds are those in which the long-chain alkyl group is a chain of 12 to 16 carbon atoms, while the less effective are those of lower or higher molecular weight. The germicidal efficacy of many organic compounds is similarly greatly affected by chemical structure. For example, in compounds such as alcohols, resorcinols, etc., it increases with molecular weight up to a certain point beyond which it decreases with increased molecular weight.

There are many detergent-disinfectants now available, and more are being marketed constantly. It is not necessary to discuss all of them here. Each has its own advantages and disadvantages. As a class, the quaternary ammonium halides have certain important properties in common. They are:

- (1) microbicidal (or bacteriostatic?) in fairly high dilutions;
- (2) not highly irritating or toxic;
- (3) not corrosive and do not stain or discolor;
- (4) not ill-tasting or malodorous;
- (5) readily soluble;
- (6) stable per se but are partly inactivated in the presence of organic matter or colloids;
- (7) relatively inexpensive;
- (8) powerful surface tension reducents; therefore good wetting agents.

Their microbicidal action may be more apparent than real as they are probably very effective bacteriostatic agents. Furthermore, caution is necessary in interpreting the test data, because at least some of them have the property of causing microorganisms to become sticky and to stick to solid objects, and together in clumps. This produces an apparent reduction in numbers, partly through agglutination and not entirely through actual killing. However, tinctures‡ of quaternaries appear to be very effective indeed.

The mechanisms of the antimicrobial actions of quaternaries are not completely understood but are believed to be analogous to the action of sulfonamides, antibiotics, etc., i.e., they may act as specific enzymic poisons. They must also have marked occluding or smothering effects because of their tendency to accumulate on cell surfaces. One other very striking effect is *lysis* of many species of bacteria. This will be discussed in more detail later.

**CATIONICS, ANIONICS AND NON-IONICS.** There are three types of these compounds: (1) those in which the organic radicle is a cation (quaternary

\* *Detergent* is from the Latin *detergere*, to wash away.

† They are known in the industry as "quaternaries" or, more simply, as "quats."

‡ Solutions in alcohol.

ammonium halides); (2) those in which the organic group is the anion, e.g., sodium-lauryl-sulfate, and (3) those which do not ionize. These compounds are, therefore, classified as *cationic*, *anionic*, and *non-ionic*, respectively (Fig. 18-1). The cationic compounds tend to react with anions, and vice versa. The cationic compounds appear to be more generally effective as disinfectants. The non-ionic compounds do not ionize in water. They are often used as adjuncts in commercial detergents.

**Concentration, Time, Temperature and pH or pOH.** Disinfectant solutions are not magical. Their values differ in different situations, depending on the kind of bacteria to be destroyed and their location (open wounds, sewage, skin, floors, dishes, clothing). No matter what disinfectant is selected for use under any given circumstances, at least four important factors besides surface tension play a part in the results. These are:

I. CONCENTRATION. In general, the more concentrated a disinfectant, the more rapid and certain its action.

The effectiveness is, however, not linearly related to concentration but exponentially. For example, doubling the concentration of phenol in a solution does not merely double the killing rate for bacteria but may increase it by as much as 98 per cent. Thus, concentration of disinfectant beyond a certain point accomplishes increasingly less and is wasteful.

The same effect as concentration may often be obtained by adding to a disinfectant solution some reagent (e.g., NaCl) which tends to push the disinfectant out of solution, i.e., the saturation point is reached so far as the disinfectant substance is concerned. It then tends to accumulate elsewhere, such as in the cell or on its surface.

When a disinfectant is in a colloidal state (emulsion) the material in each minute globule is highly concentrated. The bacterial cell in contact with these colloidal globules is, therefore, in contact with a high concentration of disinfectant.

**OLIGODYNAMIC ACTION.** It is well known that many substances which are toxic in relatively large amounts actually stimulate growth at certain low concentrations. To illustrate, if a bright piece of a heavy metal, as copper, silver or gold, is placed on a plate of extract agar previously heavily inoculated with an organism such as *Micrococcus pyogenes*, and the plate incubated, small quantities of the metal diffuse into the agar and inhibit growth of the organisms in a zone around the metal piece. This phenomenon is ascribed to what is called *oligodynamic\* action* of the metals (Fig. 18-2).

At the periphery of this barren zone one might suppose that, as the concentration of metal ions diminishes with distance from the metal piece-

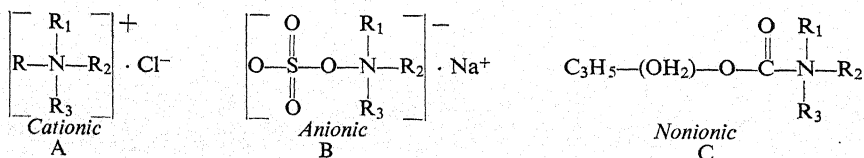


Fig. 18-1. Diagrams of the structure of three types of quaternary compound. For explanation see text.

\* Action in minute concentrations.



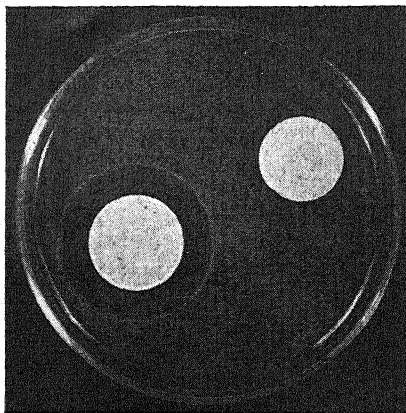


Fig. 18-2. Biological effect of minute amounts of certain metals (oligodynamic action). The agar medium was heavily inoculated with micrococci while warm, well mixed, and poured into the plate. When solid, the metal disks (coins) were placed on the agar surface and the plate was incubated. The bacteria grew where they could, producing a greyish, granular appearance. Note that the silver disk (quarter) is surrounded by a clear zone where no growth occurred, while the nickel disk shows little or no zone of inhibition. Note also the increased density of the growth at the outer margin of the clear zone around the quarter. This may be due to: (a) less competition for food at the edge of the sterile area; (b) stimulation by a critically small concentration of the metallic ions; or (c) both. The difference in action between nickel and silver is not necessarily a general one. With another species of test organism the situation could be reversed. Further, the coins are not pure silver or nickel. (Photo courtesy of Dr. Walter C. Burkhardt, Department of Bacteriology, University of Georgia, Athens, Ga.)

growth would increase: slightly, close to the zone; more and more with increasing distance; and finally reach a density equal to normal in all areas beyond the zone where the metal ions had not yet migrated. On the contrary, in many cases growth is heaviest at the periphery of the sterile zone, where concentration of the toxic metal ions is minimal. Here a narrow but distinct opaque ring of extra dense growth is often clearly evident. The same phenomenon is seen in similar experiments with many other antimicrobial substances.

II. TIME. No disinfectant, as ordinarily used, acts instantly. Sufficient time for contact, and for whatever chemical and physical reactions occur, must be allowed. The time required will depend on concentration, temperature; nature of the organisms; existence in the bacterial population of cells having varying susceptibilities to the disinfectant due to different ages and other physiological factors, mutation, etc.

RATE OF DISINFECTANT ACTION. As in sterilization by heat, chemical disinfectant action is an orderly, continuous process. Under ideal and uniform conditions of temperature, concentration of disinfectant, pH, etc., the rate at which death of the organisms in a pure culture occurs is often constant and the number alive at any moment is *theoretically* a function of time only. In such cases the rate of death suggests that the disinfectant is reacting with molecules of a single substance in organisms of identical susceptibility to the disinfectant, under the laws of mass action—a *monomolecular reaction*. Theoretically in such cases, if the logs of the number of surviving organisms

are plotted against units of time, a straight line should be obtained. If this line is nearly perpendicular obviously the *rate* of killing is high; if it slopes gently, the rate is low. The rate (high or low) under ideal, theoretical conditions, tends toward *uniformity* throughout and in such cases the *slope* of the line is affected only by such extraneous factors as temperature, pH, and concentration of disinfectant (Fig. 18-3).

Unfortunately, disinfectant action is not so simple. The *form* of the curve, and also its slope, are dependent to a large extent on the species of organism and on the presence of some cells which are more resistant or more sensitive than the majority, to the disinfectant. Usually both sorts of cells are present. Usually, also, several kinds of reaction occur simultaneously; coagulation; non-specific chemical combinations, various enzyme blockages, etc. At times the reaction between disinfectant and organism may proceed as a bi, tri-, or quadrimolecular process or one of an even higher order, or mixed orders, suggesting that (a) the reactions between protoplasts of different species and different disinfectants are very complex, and (b) what appears to be a reaction of a monomolecular type probably is the algebraic sum of a number of simultaneous reactions, the net result of which superficially resembles a monomolecular reaction. Curves representing numbers of organisms surviving at any moment may not be straight lines but deflected in various ways due to factors already mentioned and others not fully understood.

Of course, if very high concentrations of disinfectants, or very high temperatures, are applied, all of these gradual depopulations and measurable fluctuations are masked and lost in one instantaneous, catastrophic stroke.

III. TEMPERATURE. As a rule, the warmer a disinfectant, the more effective it is. This is based partly on the principle that chemical reactions in general are speeded up by raising the temperature. As a rule, within ranges near the

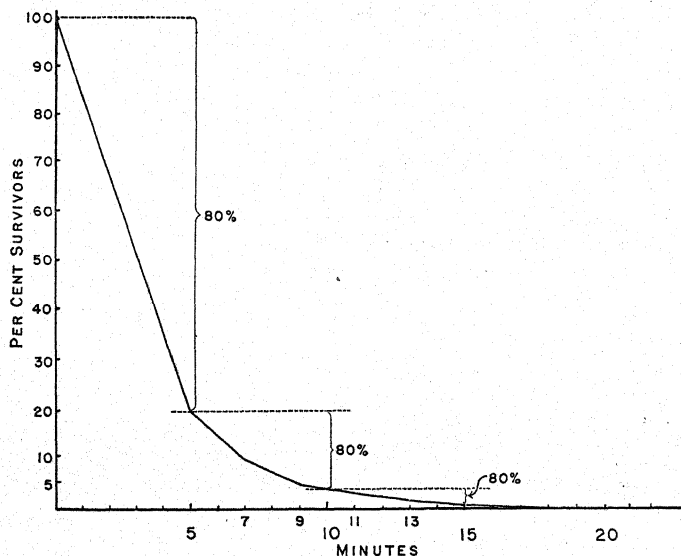


Fig. 18-3. Relation between time and death of organisms in a situation in which the lethal agent acts at a constant rate under uniform conditions.

lethal point for microorganisms and below, a rise in temperature of  $10^{\circ}\text{C}$  increases reaction rates two to eight times. This is particularly true of coagulation by hydrogen ions.

However, since many disinfectant actions are partly physical in character, the laws governing chemical reactions do not apply exclusively. Higher temperatures generally lower surface tension, increase pH, decrease viscosity, diminish adsorption and produce combinations of effects which are little understood.

**IV. pH AND pOH.** As a general rule, the lethal or toxic action of harmful agents, both physical and chemical, is affected by  $(\text{H}^+)$  or  $(\text{OH}^-)$ . The synergistic relationship between heat and  $(\text{H}^+)$  has already been discussed. Heat tends to cause greater dissociation of acids. The activity of phenol is markedly increased by increases in hydrogen ion concentration.

The increase in effectiveness of benzoates and salicylates in acid solution is an excellent example of the effect of  $(\text{H}^+)$  on antiseptic action (see Chapter 43). In the case of these and other weak organic acids, the undissociated molecule, and not the anion per se, is the active agent. The effect of increased  $(\text{H}^+)$  is to suppress ionization of the weaker acid, thus increasing the concentration of the undissociated toxic molecules. Probably  $\text{H}^+$ ,  $\text{OH}^-$ , and heavy-metal cations are the most important ions in relation to disinfection by chemical agents.

**Inactivation of Antimicrobial Agents.** NON-SPECIFIC ORGANIC MATTER. Most common disinfectants are quite general in their affinity for protoplasm, protein, etc. Substances like phenol, bichloride, strong acids, chlorine, etc., tend to combine with and destroy or coagulate *all* proteins or colloids. The presence of considerable quantities of proteins or colloids such as blood serum, mucus, feces, etc., in any material being disinfected will, therefore, protect the organisms to a great extent by combining with the disinfectant before it reaches the organisms. This is an important point to remember. In addition to combining chemically with such organic substances the disinfectant is to a great extent removed by them. These substances (proteins, mucus, feces, blood, etc.) are largely colloidal. The colloidal structure presents a very large surface at which surface-active disinfectants are adsorbed and removed from the bacteria, even after coagulation has occurred.

**CHEMICAL ANTAGONISMS.** Many antimicrobial agents may be inactivated by the extraneous presence of certain specific substances with which they react readily. Simple examples are the inactivation of acid by the presence of a carbonate or hydroxide; the precipitation of toxic sodium oxalate by calcium chloride. These are specific inactivators. Bichloride of mercury, while exerting *irreversible coagulative* effects in high concentrations (1:1000 or stronger), exerts a *specific* and reversible chemical effect in dilutions of 1:100,000 or more. The toxic action of dilute mercuric chloride is due to specific combinations with the sulfhydryl ( $-\text{SH}$ ) group in vital portions of the cells. The  $-\text{SH}$  group is a very important, functioning part of many enzymes. When one enzyme is stopped or blocked a whole series of dependent enzyme reactions may also stop, both above and below the blocked enzyme. Mercury may thus be viewed as a *specific protoplasmic poison*. The toxic action may be completely neutralized by putting  $-\text{SH}$  compounds such as glutathione and cysteine into the solution. These combine with the  $\text{HgCl}_2$ . *Micrococcus pyogenes* in contact

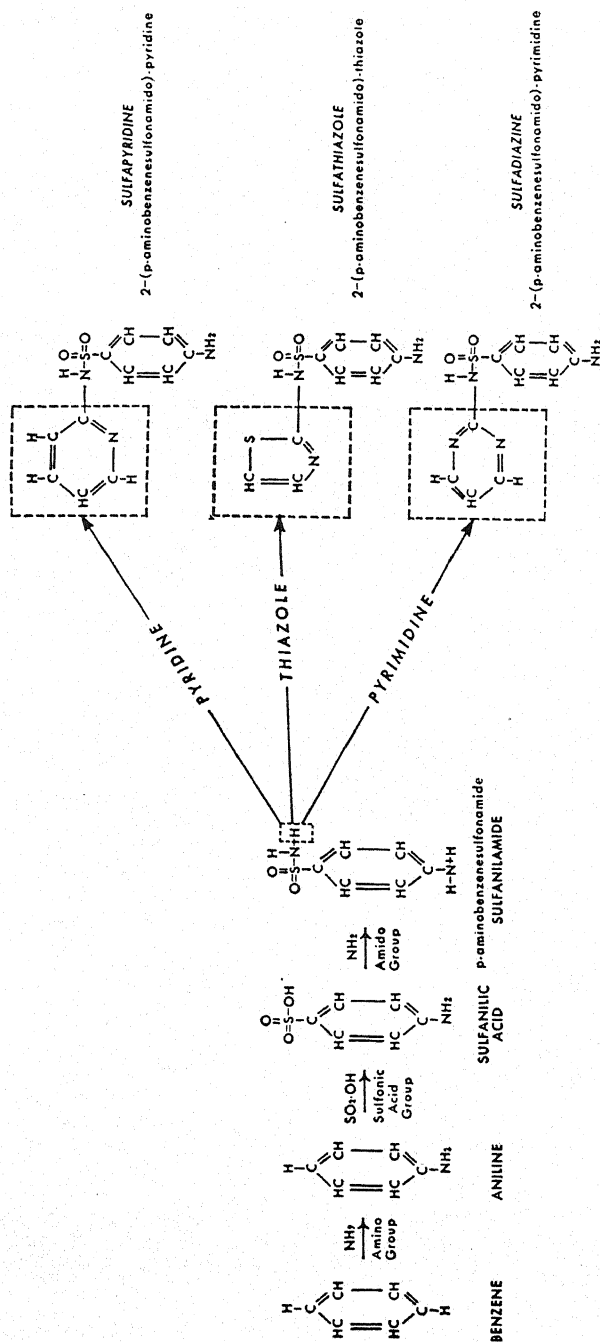


Fig. 18-4. How the sulfonamide drugs are derived from benzene. First, an amino group is added, yielding aniline (a base from which dyes are synthesized). The addition of a sulfonic acid group to aniline yields sulfanilic acid. The addition of another amino group yields sulfanilamide. The addition to this, in place of one hydrogen atom, of various other chemical groups, yields other sulfanilamide drugs, three of which are shown in the diagram.

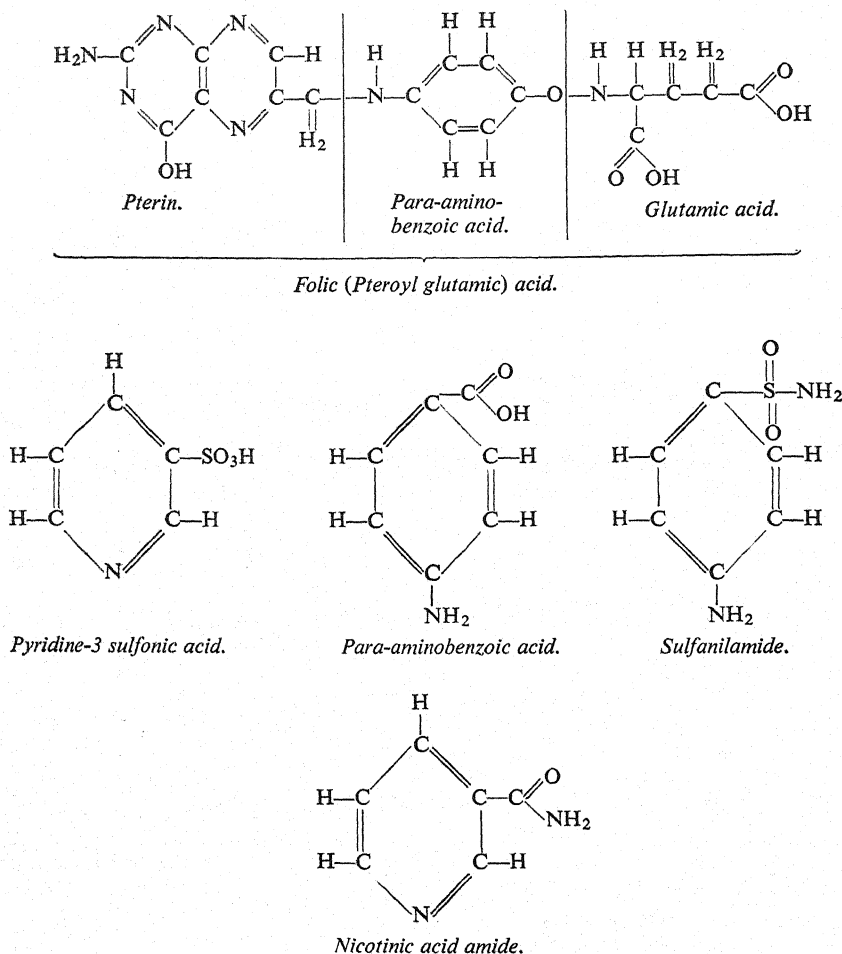



Fig. 18-5. Diagram showing the molecular structure of several important vitamins, and of some drugs used in chemotherapy. Note the similarity between sulfonamide, the drug, on the one hand, and the vitamins: nicotinic acid amide and para-aminobenzoic acid, on the other hand. Note also the position of para-aminobenzoic acid in the molecule of the folic acid molecule. For explanation see text.

for 72 hours with 0.001 per cent  $\text{HgCl}_2$ , and apparently dead, can be "revived" by treatment with  $\text{H}_2\text{S}$ , which precipitates the  $\text{Hg}$  as  $\text{HgS}$ . The lethal action of dilute phenol on micrococci and on *Salmonella typhi* may be stopped and the apparently dead organisms "revived" by removal of the phenol with activated charcoal or ferric chloride. In Chapter 12, we have seen how micro-organisms apparently dead due to ultraviolet irradiation can be reactivated by visible light and other agents.

**THE SULFONAMIDE DRUGS.** The sulfonamide drugs are synthetic compounds all based on the same central molecule, sulfanilamide:

$\text{NH}_2$    $\text{SO}_2\text{NH}_2$ . The amino group ( $\text{NH}_2$ ) in the ortho position (shown at left in the diagram above) is most important. (See also Fig. 18-4.)

Strictly speaking, the sulfonamides include only those derivatives of sulfanilamide which structurally so closely resemble para-amino benzoic acid (PABA) (Fig. 18-5) that they can *antagonize* that substance; that is, they can act as a sort of counterfeit PABA. It appears that they can take the place of PABA in a vital, enzymic function in the bacterial cell but, being physiologically inert in that situation, the enzymic function is blocked.

Let us suppose a series of synthetic reactions in a cell is carried on by a series of enzymes A, B, C, etc. (Fig. 18-6, series I). Let us suppose, also, that enzyme C is one which contains an absolutely essential, functional group called folic acid.\* Now, looking more closely at a graphic formula of folic acid (Fig. 18-5) we see the molecule PABA occupying a prominent place in it. Comparing the formula of PABA and sulfanilamide it takes no Sherlock Holmes to see the similarity between the two and the possibility that a sulfonamide drug can play the imposter and take the place of PABA in the folic acid molecule.

Enzyme C, then, is one which depends on folic acid and this, in turn, on PABA to function. The result of the blockage of folic acid in enzyme C is a pathologic accumulation† or “backing up” of partly completed metabolic products at enzymes A and B. A complete stoppage of D, E, and F results through lack of material from C on which they can work. The entire enzymic production line ceases to operate.

CHEMOTHERAPY. Referring again to our production line analogy, let us suppose that we replace the “dummy” (sulfonamide) at C with the functioning agent by (1) adding enough PABA (the essential metabolite) to displace and exclude the sulfonamide. This is chemotherapy. It also illustrates the principle of *metabolite antagonism*.

We may also offset the blockage at C by (2) supplying complete folic acid, or (3) supplying, in the culture medium, the product that would have been formed by C (or any subsequent enzyme in the chain) or, (4) supplying the finished product of the enzyme series.

The cell itself may be quite capable of doing this very thing. For example, let us suppose that in a parallel series of enzyme reactions (series II, Fig. 18-6) some of the substance usually made by C is produced as a side reaction or intermediate stage in the function of H. This product becomes available to D—and voilà! Such a cell is more or less *sulfonamide-resistant*, depending on how much of the product needed by D is available from H.

ENZYME SERIES I : A → B → C → D → E → F →

ENZYME SERIES II : G → H → I → J → K → L →

Fig. 18-6. For explanation see text.

\* This is an important vitamin, necessary for synthesis of the amino acids methionine, serine etc., which go into the synthesis of protein and this, in turn, into the making of protoplasm. Such absolutely essential specific substances, whether vitamins, amino acids or other compounds necessary to the completion of a particular functional part of the cell, are called *essential metabolites*.

† Examples of such accumulations are dietary (vitamin) deficiency diseases such as *scurvy* (vitamin C or ascorbic acid) and *beri-beri* (vitamin B or thiamine).

Numerous other such by-passings are known to occur.

**DRUG-FASTNESS.** If certain microorganisms are kept in contact with small, sub-lethal concentrations of toxic substances (various salts, phenol, antibiotics, sulfa drugs, etc.), the organisms often become highly resistant to the effects of toxic substances. Drug-resistant organisms are often designated as *drug-fast*. As indicated in the preceding paragraph, drug-fast strains of microorganisms are generally thought, on very sound experimental grounds (Newcomb's experiment; Luria-Delbrück experiment, etc.), to represent survival and growth of resistant mutants pre-existing in the exposed population in imperceptible numbers. These dominate the microbial population after the susceptible majority die off. The drugs themselves appear rarely to *induce* the mutation to resistance.

**DRUG DEPENDENCE.** The appearance of strains wholly dependent on drugs seems as strange as the emergence of resistant strains. The two are probably not related except that they are two manifestations of the phenomenon of variation. Dependent mutants obviously could not survive and develop significantly except in an environment containing the factor on which they are dependent. It is only because we have tried to cultivate microorganisms in contact with antibiotics that these curious mutant forms have been revealed.

It is conceivable that strains dependent on all sorts of other unsuspected substances (such as unicorn's horn or powdered emeralds ?) are also constantly occurring, but we never learn about such strains because we never think to test for the presence of such curious organisms by growing our cultures in media containing unicorn's horn or pulverized jewels. However, we might profitably try various amino acids, traces of certain metals, lipids, etc. Some valuable new organisms might be found. This sort of investigation is like treasure hunting.

The mechanism for dependence is not entirely clear. Dependence may result from the absence, in some mutants, of certain molecular gaps in key enzymes, which can be filled only by certain molecular groupings in the drug on which the microorganism is dependent. Such organisms are veritable microbial drug addicts!

**CHEMOTHERAPY AND CANCER.** A very interesting aspect of chemotherapy concerns the possibility of selectively poisoning the cells of neoplasms. The cells of neoplasms are thought to have metabolic mechanisms different from those of normal cells. The obvious possibility presents itself of finding some metabolite antagonist which will poison an enzymic mechanism peculiar to the neoplasm cell and not present in the normal cell. Thus, it is conceivable that we could cure cancer overnight by means of a few tablets or injections of some antibiotic-like drug.

This rosy dream may not be too far from reality. Stranger things have happened. Already some drugs have been found which, if not the whole answer, at least point definitely to the possibilities in research on cancer metabolism. Fame, Fortune and Honor are to be won in this field!

#### THE EVALUATION OF DISINFECTANTS

One often hears a disinfectant spoken of as "strong" or "weak" or "mild." These terms are inexact and convey different meanings to different people. To one they mean a "disinfectant odor"; to another, pain on application to

a scratch; to still another, corrosive action; to another a definite color. Rarely does the untrained person think of disinfectants in terms of *microbicidal* activity, or *toxicity* for human beings or animals. He has to judge them mainly by subjective criteria. Actually, the value of any substance as a disinfectant depends on a number of factors, important among which are: the microorganisms concerned; the effect of the disinfectant on various materials such as discoloration, corrosion, irritation of tissues, and toxicity in contact with the body. Surface tension, cost, effectiveness or bactericidal action under the conditions surrounding its use, stability and odor are also important factors. Not all disinfectants are equally effective, and some of them are more effective against some bacteria than against others. Some are effective in pure cultures in the test tube but not in contact with organic matter like blood, feces or dead tissues; some are effective in the vapor phase but not as liquids (see Chapter on Microbiology of the Atmosphere).

The effectiveness of many disinfectants may be estimated by mixing them, in certain concentrations, with cultures of certain bacteria and measuring the time required for the substance to kill the organisms. If this is done under carefully standardized conditions, i.e., using a constant quantity of culture medium of a stated composition, a fixed temperature, and a suspension of measured numbers of certain bacteria of known and constant resistance, the results will be quite accurate and reproducible.

**The Phenol Coefficient.** This is based on a comparison of the effectiveness of the tested disinfectant with that of pure phenol under conditions carefully standardized as noted above. The result obtained is called the *phenol coefficient* of the tested disinfectant. In this procedure 5 ml amounts of a series of dilutions of the disinfectant to be tested (here called X) are placed in a row of tubes of standard size. A similar series of dilutions of pure phenol (here called P) of 1:80, 1:90, and 1:100, is prepared. The temperature of all is brought to 20° C in a water bath. To each tube is added 0.5 ml of a standard suspension of *Salmonella typhi* having a resistance to phenol such that it is killed in ten minutes by the 1:90 dilution. At intervals of 5, 10 and 15 minutes, a standard loopful (4 mm loop of no. 23 B and S wire) is transferred from each tube, in succession, to tubes containing 10 ml of sterile broth of standard composition.

After 48 hours of incubation at 37° C growth in the broth tubes is recorded. The phenol coefficient is the ratio of the highest dilution of X not killing the organisms in five minutes (evidenced by growth in the corresponding broth tube), but killing in ten minutes (evidenced by no growth in the broth), to the corresponding dilutions of P. The values obtained are shown in Table 10.

In this experiment, the 1:90 dilution of P failed to kill in five minutes but killed all the *S. typhi* cells in ten minutes. This is compared with X which did the same in a dilution of 1:450. The ratio of X to P is  $\frac{450}{90}$  or 5, the FDA\* phenol coefficient of X. The determination of phenol coefficients requires a high degree of skill.

The significance of a phenol coefficient has very definite limitations. For example, a disinfectant dissolved in distilled water may have a phenol co-

\* U. S. Food and Drug Administration.



efficient as high as 50. However, it may be wholly ineffective if applied in the blood, or used in contact with matter such as pus, saliva, feces, milk, etc., as these may combine with the disinfectant and remove it from the bacteria. Further, it may have a coefficient of only 2 or 3 when tested against some other organisms, such as *Micrococcus pyogenes*. When a substance is said to have a certain phenol coefficient, the limitations of the method must be kept in mind.

Other factors affecting the phenol coefficient are the composition and pH of the broth and variations in the organism. Most important, since the comparison is made with pure phenol, the method is properly applicable only to

**Table 10.** Typical Data from a Phenol Coefficient Determination.

DISINFECTANTS	DILUTIONS	5 MINUTE SUBCULTURES	10 MINUTE SUBCULTURES	15 MINUTE SUBCULTURES
Phenol	80	— *	—	—
	90	+ *	—	—
	100	+	+	+
"Unknown"	350	—	—	—
	400	+	—	—
	450	+	—	—
	500	+	+	—
	550	+	+	—
	600	+	+	+
	650	+	+	+

\* + = growth; — = no growth.

water soluble substances chemically related to phenol or acting on micro-organisms in the same way. However, in practice, many sorts of disinfectants are (often improperly) evaluated in terms of phenol coefficient.

In order to overcome some of these difficulties the Food and Drug Administration Laboratories make tests of commercial disinfectants with cultures of various organisms and under circumstances designed to imitate actual situations. Thus, tests are often conducted in the presence of blood serum. For testing insoluble or oily products, special methods are used which are intended to approach practical conditions of use. Among these are the filter paper methods (wet and dry) and the agar plate (plain and cup) methods.

**Inactivators.** It is important to note that many substances do not *kill* in ten minutes but merely prevent growth of the test organisms in the broth subcultures. The apparent killing and seemingly very high phenol coefficient by some mercurials, for example, can be prevented if the transfers from the dis-

infectant tubes are made to broth containing thioglycollate. Thioglycollate inactivates the mercury. Growth may then occur in all tubes and the phenol coefficient is nil. This is a good example of (1) bacteriostasis and (2) reactivation by removal of the poisonous substance. With substances like  $\text{HgCl}_2$ , the quaternaries, and the sulfonamides, the active agent is closely attached to, or inside of, the cells and cannot be removed or stopped by dilution. A definite antagonist or inactivator is used. For sulfonamides para-aminobenzoic acid has been mentioned, as have sulfur compounds for  $\text{HgCl}_2$ . For quaternaries no entirely satisfactory inactivator is available although "Luramin sodium," combined with "Tween-80" and Azolectin and other substances have been found useful.

**The Use-dilution Test.** No single method of testing gives a complete picture of the advantages and disadvantages, or actual value, of any given disinfectants under all conditions of use. In practice it has been the custom to use a disinfectant in a dilution (*use-dilution*), represented by 20 times the phenol coefficient. If the phenol coefficient is 5, the substance is arbitrarily used in dilution of 1:100. This was formerly thought to provide a wide margin of safety.

**THE USE-DILUTION CONFIRMATION TEST.** In an attempt to give some further idea of the activity of disinfectants under actual conditions the *use-dilution confirmation test* was devised. In this test small, polished, steel cylinders (about 7 x 2 mm) are dipped into a young broth culture of *Salmonella choleraesuis* (a typhoid-like bacillus) and then dried for about 60 minutes at 37° C. Of these contaminated rings, one is placed in each of 10 tubes containing the disinfectant being tested diluted to 100 times its phenol coefficient. The temperature is held at exactly 20° C. After exactly 10 minutes the rings are removed and transferred to broth culture to see if any of the organisms originally dried upon them remained alive. An inactivator of the disinfectant is included in this culture, to prevent any continued lethal or bacteriostatic action of the disinfectant which might still adhere to the steel cylinder.\*

Some interesting data were collected by this method. Some disinfectants with phenol coefficients of 5, ordinarily used in a dilution of 1:100, were found to have a steel-ring use-dilution of 1:100 vs. *S. choleraesuis* and 0 against *Micrococcus pyogenes*. Others, with a phenol coefficient of 2.5 and ordinarily used in a dilution of 1:500, were found to have steel-ring use-dilutions of 0 against both *S. choleraesuis* and *M. pyogenes*.

Modifications of this use-dilution method have been devised for testing disinfectants under conditions of actual use, such as on steel surfaces, linoleum, asphalt tile, etc. Such data are invaluable in the hands of hospital and institution administrators, sanitary engineers, etc., who must provide satisfactory disinfection procedures on a large scale.

Many other methods of evaluation have been described. (See references.)

**Toxicity of Disinfectants.** One of the great problems in the selection and use of disinfectants for use in contact with living tissues (i.e., for superficial applications) is the avoidance of excessive toxic effects. Several methods are

\* If no inactivator is used, the cylinders are dipped into a preliminary tube of broth to remove as much disinfectant as possible before putting them into the final culture tube. Unless all ten cylinders are sterilized by the disinfectant, certification of that dilution of the disinfectant as a proper use-dilution is withheld.

available to test the toxicity of disinfectants. The results are sometimes expressed as *toxicity index*, i.e., ratio of minimal toxic dose to minimal effective germicidal dose. One may determine the toxic dose of a disinfectant in various ways; for example, by observing the smallest quantity necessary to stop completely the action of phagocytes (white blood corpuscles) in a test tube in a given time. Other methods measure the inhibitory or lethal effect of the tested substance on various tissues, while still others measure the respiration quotient of tissue cells in contact with the germicides. One method is based on observing the survival time of chick embryos into which the tested substances are injected. A time-honored, simple and effective method also, for many substances, is the direct trial on living animals.

Probably no single test gives a true result, and species of bacteria, as well as of tissue cells, vary greatly in their susceptibility to different substances. For any disinfectant, the toxicity index will therefore be different for different tissues and bacteria.

#### FACTORS AFFECTING STERILIZATION BY HEAT

Heat may be applied for sterilization in three ways: (a) by steam or hot water (moist heat); (b) by prolonged baking in the oven (dry heat); and (c) by complete incineration. The last needs no comment beyond pointing out that (1) common sense will direct what may be burned up and what should not; and (2) care must be taken to see that the material is completely burned.

At the temperatures commonly used for sterilization (100° C to 170° C) several factors affect the rate at which microorganisms are killed. Important among these are the time, the temperature, numbers of organisms, pH and hydration.

**Time and Temperature.** Obviously these are inversely related. The lower the temperature, as a rule, the longer the time required to kill the organisms. At 120° C in an autoclave (compressed steam), for example, it may require only 15 minutes to kill everything, even resistant spores; whereas at 100° C in the same autoclave the same spores may survive six hours or more. Even increasing the temperature from 100° C to 105° C may diminish the killing time to two hours. The difference is due to hydration and pressure. These are discussed a little farther on.

**Numbers of Organisms.** As has been detailed in the section on disinfection with chemical agents, sterilization is an orderly, continuous, process in which there is a definite relationship between initial numbers of organisms, the efficacy of the lethal process, and the elapsed time. In other words, under *ideal* conditions, for any uniformly acting lethal agent the number of organisms remaining alive after a given interval is a function of elapsed time. This is shown on page 265.

If these relations be drawn on a chart we have something like Figure 18-3, which is a representative form of curve for many lethal agents. This shows that the majority of cells are killed quickly but that some may survive for a considerable time, depending on lethality of the agent, hydration, pH, osmotic pressure, age of cells, etc.

**pH, Hydration, etc.** We have already indicated the relation of hydration to coagulation by both chemical disinfectants and heat. In addition, heat in-

## SURVIVORS OF 100° C AFTER VARIOUS INTERVALS

Elapsed time (minutes)	Surviving organisms (per ml)
0	90 million
5	12 "
7	8 "
9	5 "
11	3 "
15	1 "
20	200 thousand
25	20 "
30	0

creases dissociation of acids (pH) and the effectiveness of ( $H^+$ ) in disinfection processes.

The close relation of pH and temperature in lethal processes is illustrated by the following:

**Table 11.** *Relation of pH and Temperature to Survival of Tetanus Spores.*

pH	SURVIVAL (MINUTES) AT			
	105° C	100° C	95° C	90° C
1.2	4	5	6	6
4.1	6	11	14	23
6.1	9	14	38	54
7.2	11	29	53	65
10.2	5	11	21	24

Here it is seen that ( $OH^-$ ) is also an adjuvant of heat, but to a lesser degree than ( $H^+$ ). Acidity and alkalinity both increase the lethal effect of heat.

Fluids of high osmotic pressure tend to dehydrate the cell contents and so increase the heat resistance. The age of the organisms is also important, older cells being more heat resistant than young ones. The species or organism is a determining factor, the form of survival curve varying markedly with different kinds of organism exposed to the same lethal influence.

## REFERENCES

- Brochure. Reading on Cancer. (A Bibliography.) Pub. Health Serv. Publication No. 457, 1955. U. S. Government Printing office, Washington 25, D. C.
- Cobb, J. P.: Tissue culture observations on the effects of chemotherapeutic agents on human tumors. Tr. New York Acad. Sci., 1955, Ser. II, 17:237.
- Glassman, H. N.: Surface active agents and their application in bacteriology. Bact. Rev., 1948, 12:105.
- Jawetz, E.: Antimicrobial chemotherapy. Ann. Rev. Microbiol., 1956, 10:85.
- Julius, H. W.: The mode of action of chemotherapeutic agents. Ann. Rev. Microbiol., 1952, 6:411.
- Keller, R., and Morton, H. E.: The effect of a hand soap and a hexachlorophene soap on the cultivable treponemata. Am. J. Syphilis, Gon. and Ven. Dis., 1952, 36:524.

- Klarman, E. G.: Recent U. S. Developments in the "Official" Testing of Disinfectants. Mfg. Chemist, London, Feb., 1955.
- Lester, W., Jr., and Dunklin, E. W.: Residual surface disinfection. J. Inf. Dis., 1955, 96:40.
- Litchfield, J. H., and Ordal, Z. J.: A study of the Stuart method for the evaluation of germicides. Appl. Micr., 1955, 3:67.
- Martin, G. J.: Biological antagonism. The Blakiston Co., Inc., Garden City, N. Y., 1953.
- Moore, A. M., and Boylen, J. B.: Patterns of Growth Inhibition. J. Bact., 1952, 64:315.
- Perkins, J. J.: Principles and Methods of Sterilization. Charles C Thomas, Springfield, Ill., 1956.
- Reddish, C. F., Editor: Antiseptics, Disinfectants, Fungicides and Sterilization. Lea & Febiger, Philadelphia, 1954.
- Rhoads, C. P., Editor: Antimetabolites and Cancer. American Association for the Advancement of Science, Washington 5, D. C., 1955.
- Ritter, H. W.: The germicidal effect of a quaternary ammonium compound (cetylpyridinium chloride) on *Mycobacterium tuberculosis*. Appl. Micr., 1956, 4:114.
- Salle, A. J., and Amesur, B. R.: Evaluation of germicidal efficiencies of a group of antibiotics tested by tissue culture technique. Proc. Soc. Exp. Biol. and Med., 1956, 93:67.
- Sevag, M. G., Reid, R. D., and Reynolds, O. E., Editors: Origins of Resistance to Toxic Agents. Academic Press, Inc., New York, 1955.
- Shive, W.: Biological activities of metabolite analogues. Ann. Rev. Microbiol., 1952, 6:437.
- Spencer, S. M.: Can chemicals conquer cancer? Sat. Eve. Post, 1956, 228:32.
- Stedman, R. L., Kravitz, E., and Bell, H.: Studies on the efficiencies of disinfectants for use on inanimate objects: I, II, III, IV. Appl. Micr., 1954, 2:119, 322; 1955, 3:71, 273.
- Various Authors: Conference on Mechanisms and Evaluation of Antiseptics. Ann. New York Acad. Sci., 1950, 53:1.
- Various Authors: Symposium on gaseous ethylene oxide. Am. J. Hyg., 1949, 50:270.
- Walter, W. G.: Symposium on methods for determining bacterial contamination on the surfaces. Bact. Rev., 1955, 19:284.
- Wright, J. C.: Chemotherapy of human cancer. Tr. New York Acad. Sci., 1955, Ser. II, 17:210.

## Destruction, Removal and Inhibition of Microorganisms

### 2. PRACTICAL APPLICATIONS

#### USES OF HEAT

**A. Moist Heat:** 1. **BOILING IN WATER.** The use of boiling for preserving foods and disinfection is very simple. It is only necessary to remember that spores may remain alive even after an hour of boiling. For ordinary household purposes of *disinfection*, but not *sterilization*, five minutes' boiling of dishes, clothes, etc., is usually sufficient, provided the hot water actually comes into contact with the microorganisms, and not merely with the outside of lumps of food or packets of instruments or other objects containing bacteria, etc.

Boiling can never be depended on for *sterilization*, especially at high elevations above sea level as shown below.

**Table 12.** *Boiling Point at Various Altitudes.*

LOCATION	ALTITUDE*	BOILING POINT†
New York City.....	0	100
Chicago, Ill.....	589	98.9
Denver, Col.....	5,280	94.3
Fort Laramie, Wyo.....	7,380	92.2
Tahoe, Nev.....	10,000	89.1

\* Feet above sea level.

† Degree C

2. **FREE-FLOWING ("LIVE") STEAM.** "Live" or free steam is usually applied in a covered container which will hold steam, but without pressure. Boiling water and free steam never reach a temperature above 100° C (212° F). In mountainous regions it is lower than 100° C (see table). Free steam is sometimes used to accomplish *fractional sterilization* or *tyndallization*.

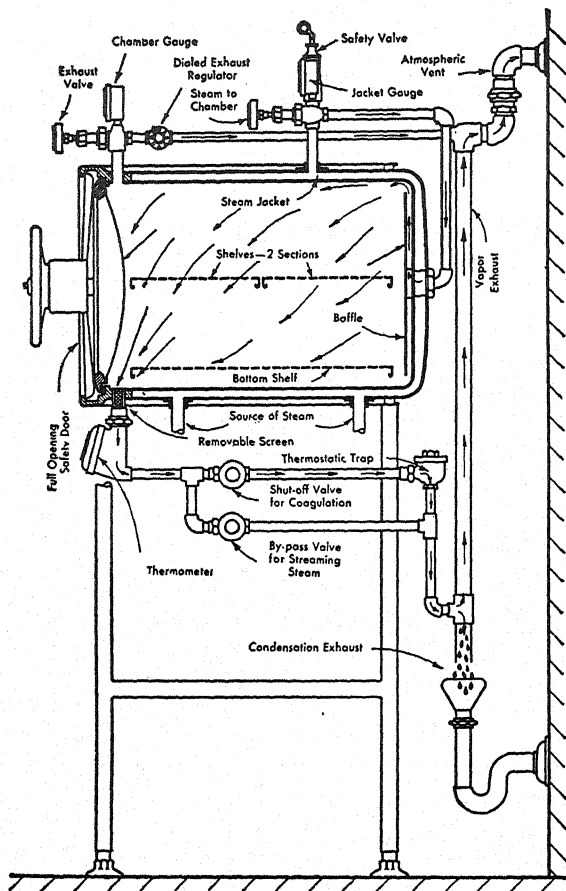


Fig. 19-1. Diagrammatic illustration of steam jacketed autoclave. (Surgical Supervisor, Dec., 1946. American Sterilizer Co.) Steam enters the *jacket*, a double-walled shell, at two places just beneath the cylinder. It passes out the top through a pipe to which are attached: a wheel valve admitting the steam to the inner *chamber*; a *safety valve*; a gauge showing *pressure in the jacket*. The steam enters the inner chamber at the right of the diagram, filling the upper portion. Its pressure registers on the *chamber gauge*. It may be allowed to escape rapidly by the *exhaust valve*. If this is closed, steam pushes the cooler air in the lower portion out at the bottom (left), where the *thermometer* registers *proper temperature only* when the air is gone and is followed by the hot steam. The escaping steam may be allowed to flow out without building up any pressure if the *by-pass valve* is fully opened. If the by-pass valve is closed and the *shut-off valve* is opened, steam passes through the *thermostatic trap* where the heat shuts off all but a pinhole opening. This causes pressure to build up in the chamber, yet *prevents stagnation* by permitting a constant *minute flow* of steam through the apparatus.

**TYNDALLIZATION.** Tyndall, a famous British scientist, noticed that a period of about twenty-four hours at ordinary room temperatures is usually sufficient to enable dormant and heat-resistant spores to germinate and to grow into the vegetative state in which they are sensitive to heat. He devised a process of sterilization based on these observations. It consists in steaming for a few minutes at 100° C on three or four successive occasions, separated by twenty-

four-hour intervals at a temperature favorable to spore germination. The intervals permit the dormant, resistant spores to become active, vulnerable, vegetative cells, readily killed by 100° C. This process renders an infusion sterile whereas one single, continuous boiling for one hour may not, since many spores remain in their dormant and resistant state during this time. This intermittent process is put to practical use in the home canning of foods. A disadvantage of the method is the time required to achieve complete sterilization. An advantage is that it requires no special apparatus. In some fluids, as water, spores may not germinate promptly. Also, if the material is freely exposed to air, anaerobic spores may not germinate and may survive the process. If not freely exposed to air, aerobic spores will not germinate freely.

3. COMPRESSED STEAM. AUTOCLAVING. Anyone familiar with the operation of a home pressure cooker is familiar with an *autoclave* because the cooker is a simple form of autoclave. In the autoclave (Fig. 19-1), be it a small and simple home pressure-cooker or an apparatus for a hospital, large enough to fill a room and fitted with various gauges, pipes, valves, clocks and wheels, the object of both is alike: to heat the articles to be sterilized by means of steam under considerable pressure.

Steam under pressure is hotter than boiling water or free-flowing steam such as is used in tyndallization. The higher the steam pressure, the higher the temperature.

The relation of *steam* pressure to temperature is shown in the following table:

**Table 13.** *Relation of Temperature and Pressure of Pure Steam.*

Temperature °C (at sea level)	Pressure (Pure steam)
100.....	0
109.....	5
115.....	10
118.....	12
121.....	15
124.....	18
126.....	20

It must be remembered that it is the compressed *steam* (moisture, *hydration*) that sterilizes and not compressed air (dry and usually not as hot as steam).

For example, pure steam at 15 pounds pressure has a temperature of 121° C. If the steam is mixed with an equal amount of air, at the same pressure the temperature is only 110° C while if the mixture is 2/3 air, the temperature is only 109° C.

Steam *hydrates* and thus promotes coagulation. Air does neither. Steam, being water vapor, also produces *hydrolysis* at autoclave temperatures. Dry air cannot do this at any pressure or temperature. In autoclaving, therefore, as in using a pressure cooker, a valve is left open for the escape of all air *before the steam pressure is allowed to rise*.

The actual amount of water present as steam in an autoclave is small and articles soon dry off after removal, especially if removed from the autoclave while hot.



The *thermometer* on the autoclave is the important guide to the process of autoclaving, not the pressure gauge. However, the latter, as well as a steam-escape or safety valve, is essential to safety.

The common practice in autoclaving fluids or freely exposed surfaces such as those of dishes and instruments is to apply 115° to 125° C (10 to 20 pounds' pressure) for twenty minutes. The pressure must be allowed to subside *slowly* after the heating is over or the *superheated* fluids in the vessels will boil over. Any large, solid masses must be heated a longer time, to allow for heat penetration. Packages must be spaced so as to allow free circulation of steam. Substances like mineral oil or petrolatum, sand, etc., or any *dry* objects in tight jars, or which are *impervious to moisture*, cannot be satisfactorily sterilized in the autoclave. The *temperature* may rise as high as 125° C, but, in the absence of moisture, this is ineffective. Such materials are more effectively sterilized by dry heat in an oven.

Acid materials, canned tomatoes, acid fruits, pickles, sauerkraut, etc. (pH more acid than 4.5) require much shorter periods and /or lower temperatures for sterilization than low acid foods (pH 6.5 to 7) like milk, corn, meats, etc. This is because of the synergistic action between pH and temperature.

**B. Dry Heat.** Dry heat is used in oven sterilization. It is necessary to bear in mind that significant coagulation does not occur when moisture is not present.

Articles in ovens are very dry and therefore, in order to be freed of live spores, must reach a very high temperature (165° to 170° C; 329° to 338° F). It is customary to apply 165° C for a period of two to three hours. This accomplishes coagulation but, what is more effective, slight charring.

A home oven can easily be used for sterilization. A moderate temperature (330° F) is satisfactory, and the heating should be allowed to proceed for three hours after reaching that temperature. Paper wrappings should be slightly browned but not brittle; muslin or string should be yellow, due to the heat.

Only dry articles not injured by baking (glassware, bandages, instruments, mineral oils, petrolatum, and the like) may be thus sterilized. Solutions containing water, alcohol or other volatile substances will, of course, boil away and be ruined.

**Thorough Heating Necessary.** In any process of disinfection or sterilization by heat it is *absolutely essential* that the object be heated through and through and that the *center* of the object be held at a killing temperature long enough to destroy the bacteria.

Thus, in canning, a quart jar of spinach may be held in free steam (100° C) for five to ten minutes and when grasped with the hand will feel very hot. However, large masses of non-fluid materials like quart jars of canned vegetables and roast meat, in which the contents cannot *circulate*, require a long time (1½ to 2 hours), even in the autoclave, to be heated so thoroughly that the center reaches a sporicidal temperature. Pieces of meat or vegetables to be sterilized in jars by heating should be loosely packed, allowing space for circulation of the fluid in the jar. Air pockets in the jars should be carefully removed. Penetration of the heat is facilitated if the cans or jars or pieces of roasting meat, etc., be small and not packed too closely together, promoting free circulation of steam or hot air around and between them.

## STERILIZATION BY FILTRATION

**Clay and Paper Filters.** An exceedingly useful method of freeing fluids from bacteria is by means of filtration. Specially graded and purified clays, and fine unglazed porcelain are used commonly. For certain filters (Seitz, etc.), compressed paper or asbestos disks take the place of clay. In some filters the clays or porcelain are shaped into convenient, hollow cylindrical forms, closed at one end, and mounted on funnel-shaped stems. They are sterilized by heat before use, so that bacteria in the filter or glassware will not contaminate the *filtrate* (filtered product).

Various forms of laboratory filter are used, one being shown in Figure 19-2. A convenient filter, using compressed paper disks as the filtering agent, is the Seitz type (Fig. 19-3).

**Membrane or Ultra-Filters.** A particularly useful and versatile type of filter is the so-called membrane filter made of collodion, cellulose acetate, or similar substance. A common form is a disk about 50 mm in diameter and 0.1 mm in thickness. It has very fine, tubular openings from upper to lower surface. These may be varied in diameter by the manufacturer from over 1 micron to less than  $0.005\ \mu$ . These filters permit aqueous fluids to pass freely but withhold microorganisms in the fluid.

A particular advantage of the membrane filter is that all of the microorganisms in a relatively large volume of fluid (water, milk, urine, diluted blood, etc.) may be collected on one small disk where they may be observed directly or cultivated in situ. There is no need to handle hundreds of tubes or flasks with large-volume cultures. Uses of these filters have been mentioned in Chapter 13.

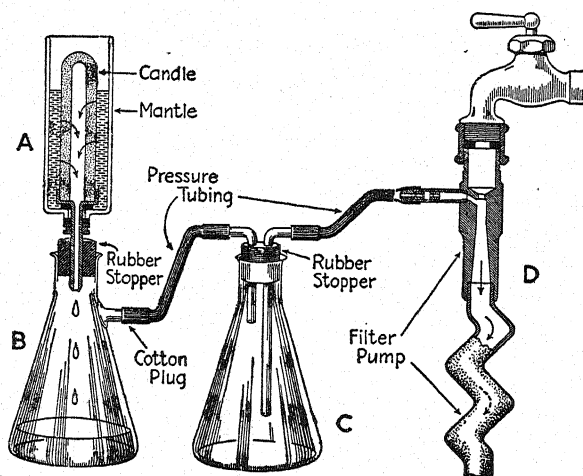


Fig. 19-2. Typical filtration apparatus. The hollow "candle" of filtering clay, surrounded by the fluid to be filtered, enclosed in a glass cup or mantle, is seen at A. The fluid passes through the clay and is drawn into the flask B by suction developed by the pump D. The flask C is to trap any fluid that might be sucked back from D or over from B. The cotton plug in the side arm of B is to prevent ingress of dust when B is disconnected from the tubing. (Belding and Marston, A Textbook of Medical Bacteriology, D. Appleton-Century Co., Publishers.)

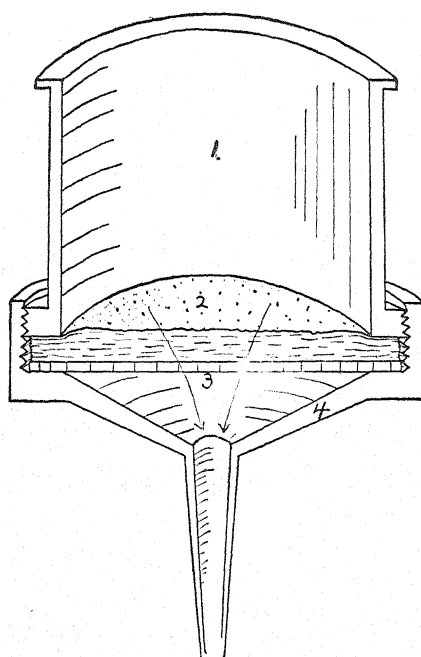


Fig. 19-3. Seitz type filter. The fluid to be filtered is poured into the cylindrical cup (1). The fluid passes through the bacteria-retaining disk of compressed paper or asbestos (2) which is supported on a wire screen (3). The stem of the funnel-shaped base (4) is inserted in a suction flask through a rubber stopper and the whole assembly sterilized before use.

### SOME USEFUL DISINFECTANTS

A few substances commonly used as disinfectants may be briefly discussed here. For purposes of discussion we may arrange them in 9 groups, as shown in Table 14.

1. **KMnO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub>.** These are mild disinfectants, sometimes used externally on tissue surfaces. They are non-irritating and of low toxicity to tissues. They are very unstable and their effect is quite temporary. They are of some value for washing deep, anaerobic wounds. Certain compounds of H<sub>2</sub>O<sub>2</sub>, such as the glycerite, are said to be of similar value in local applications.

2. **Halogens.** Iodine and chlorine are the most widely used of this group. Chlorine gas is used to disinfect filtered water at all municipal water-purification and many sewage-disposal plants. It is usually handled in tanks or tubes like oxygen, etc. It is applied to drinking water in a *final* concentration of about 1.0 part per million.

A more convenient form of chlorine for the individual and household user is calcium or sodium hypochlorite. Solutions (5%) of these are purchasable in all grocery stores under various names like *Clorox*, etc. They have a multitude of household and sanitary uses. They depend for their effectiveness probably on their liberation of free chlorine.

Various theories ascribe the antimicrobial action of hypochlorites to HOCl, HCl, "nascent" oxygen and free chlorine. Thus:  $2\text{CaOCl}_2 + 2\text{HCl} \rightarrow 2\text{CaCl}_2 + 2\text{HOCl}$ ;  $\text{HOCl} + \text{HCl} \rightarrow \text{H}_2\text{O} + \text{Cl}_2$ ; or  $2\text{HOCl} \rightarrow 2\text{HCl} + \text{O}_2$ ; or,  $2\text{Cl} + \text{H}_2\text{O} \rightarrow 2\text{HCl} + \frac{1}{2}\text{O}_2$ ; or  $\text{Cl}_2 + \text{H}_2\text{O} \rightarrow \text{HCl} + \text{HOCl}$ .

Directions for use of these solutions are on each bottle. Four points need to be remembered in their use: (1) The chlorine tends to evaporate from the solution; (2) they give a bad odor to the hands; (3) chlorine is poisonous; (4) it is readily inactivated by any and all organic matter.

CHLORIDE OF LIME (0.5 to 5 per cent aqueous solution) is excellent for similar purposes and is inexpensive. The ordinary chloride of lime of commerce is unstable and soon loses most of its free chlorine. Many organic chlorine compounds which liberate their chlorine more slowly are very effective. *Azochloramid* is one of these; *dichloramine toluol* is another. The odor of chlorine may be objectionable.

In addition to the above substances, there are many new proprietary chlorine compounds on the market based on principles given above.

IODINE is the most actively antimicrobial of the halogens. However, it is not commonly used in the same situations as chlorine because of its physical properties and its cost. Formerly used widely as a 7% tincture (alcoholic solution), this strength is far too caustic and poisonous for local application and does more harm than good. The 2% tincture is much better and is the most generally useful for *small* cuts and abrasions. If the alcohol causes too much pain, the tincture may be diluted with two to four volumes of water. An aqueous solution containing approximately 1.85% of iodine and 2.2% of potassium iodide is excellent but has a high surface tension. Gershenfeld and Witlin recommend iodine for a variety of purposes, from clinical uses to sanitization of dishes with aqueous solutions containing 200 parts per million. Alcoholic iodine, 1 per cent, is sometimes recommended for disinfecting well-wiped clinical thermometers.

Aqueous solution of iodine with added surface-tension reducent has many uses comparable to saponated cresol; for example, *Wescodyne*. Its effect, however, is transitory due to volatility of iodine.

Iodine, like chlorine, combines rather indiscriminately with organic matter; its solutions are unstable, and like chlorine, it is poisonous.

**3. Inorganic Acids and Alkalies.** These are not commonly used except in special situations where rather violent and destructive action is desired.

**4. Heavy Metal Salts.** Nearly all of the heavy metal salts have strong coagulative activity and consequently are possible microbicidal agents. However, only  $\text{HgCl}_2$ ,  $\text{CuSO}_4$  and  $\text{AgNO}_3$  are in common use.

*Silver nitrate* (1.0%) is used principally for instillation into infants' eyes at birth to prevent infection. Silver is also used as a local disinfectant in the form of organic colloidal preparations like "Argyrol" or "Protargol," in which form it is non-irritating and may be used in adult eyes at 20 per cent concentration. It is *not* for use in infants' eyes in place of  $\text{AgNO}_3$ .

*Copper sulfate* is used chiefly to control growth of algae in open water reservoirs.

*Solution bichloride of mercury* (1:1,000 aqueous) is useful for general purposes, but its strong coagulative action must be borne in mind. Like phenol, it will cause irritation and corrosion of skin, mucous membranes and wounds if left too long in contact (hence the old name "corrosive sublimate"). *All* mercury compounds are poisonous and should never be used internally except under very special conditions. The action of mercury on bacteria may

Table 14. Some Common Disinfectants.

GROUP	SUBSTANCES	PRINCIPAL ACTION	APPROPRIATE SITUATIONS FOR USE
1.	KMnO <sub>4</sub> H <sub>2</sub> O <sub>2</sub>	Oxidation.	Superficial treatment of infected tissue surfaces; lavage of infected wounds.
2.	<i>Halogens</i> Hypochlorite. (Various laundry bleaches, as "Clorox.") Pure chlorine gas. Iodine. (Iodine solutions with surface tension reductant, such as <i>Wescodyne</i> .)	Oxidation; formation of protein halides.	External: laundry, sewage, water, floors, utensils. Municipal drinking water supplies. Iodine tincture (mitis) much used for minor abrasions, etc. <i>Wescodyne</i> used for hands, skin, general household purposes.
3.	<i>Acids</i> } Inorganic <i>Alkalies</i> }	Hydrolysis; coagulation.	Not much used per se. Too destructive except in special situations.
4.	<i>Heavy metal salts</i> HgCl <sub>2</sub> AgNO <sub>3</sub> CuSO <sub>4</sub> Hg(organic)	Coagulation; specific toxic effects.	Instruments, inanimate surfaces, tissue surfaces ( <i>very dilute</i> ). (HgCl <sub>2</sub> ) (1.0%) in eyes to prevent ophthalmia of newborn. (AgNO <sub>3</sub> ) In water reservoirs to suppress algae. (CuSO <sub>4</sub> ) Organic mercurials useful in therapy and as preservatives of serum, vaccines, etc.
5.	<i>Phenol and derivatives</i> "Lysol" Saponated cresol "Hexachlorophene" Cresols, etc. Hexylresorcinol ("ST 37") Orthophenylphenol ("O-syl")	Coagulation; specific toxic effects.	Among the most generally useful disinfectants. Generally have quite low surface tensions. In saponated solutions may be used in 0.5% to 2% for hand wash, superficial wounds, objects, instruments, tables, floors, etc.
6.	<i>Radiations</i> Ultraviolet Cathode rays	Oxidation; coagulation; unknown changes; especially absorbed by nucleoproteins.	Limited use of UV to control airborne microorganisms in food establishments, packing houses, refrigerators, schools (?), hospitals (?), etc. Very microbicidal but difficult to apply in an effective manner. Cathode rays being developed for industrial use—sterilization of packaged foods, etc.

Table 14. Some Common Disinfectants (continued).

GROUP	SUBSTANCES	PRINCIPAL ACTION	APPROPRIATE SITUATIONS FOR USE
7.	<i>Alcohols</i> Ethyl-70% Isopropyl-70%	Coagulation; toxic effects(?)	Used mainly for local, mild disinfection of skin, thermometers, instruments, etc.
8.	<i>Quaternary compounds.</i>	Probably specific protoplasmic poisons. May act largely as bacteriostatic agents.	Same as phenol and derivatives.
9.	<i>Carboxide.</i>	Oxidation (?).	In special autoclaves, for materials injured by heat and/or moisture. Used mainly for special, medical or research purposes.

be reversed by iodine and some sulfur compounds provided coagulation has not occurred.

**ORGANIC MERCURIALS.** Attempts have been made to decrease the toxic, corrosive and irritating qualities of mercuric disinfectants by incorporating mercury in complex organic molecules. This has yielded a number of products which are less toxic and irritating than  $\text{HgCl}_2$  and which may even be used internally *within limits*. Some of these compounds are highly effective *in vitro*; in the presence of organic matter they are less effective but are better than  $\text{HgCl}_2$ . The value of all of this class of compounds is still in question. Other useful mercurials are phenylmercuric nitrate, very effective and of sufficiently low toxicity for *limited* internal use; and ammoniated mercury, long used as a 10 per cent ointment for external wounds, fungal infections of the skin, etc.

**5. Phenolic Compounds.** There are hundreds of derivatives of phenol which have some value as disinfectants. Their value is in great part related to their chemical structure. Some of these have uses only in special situations. The most generally useful compounds are the cresols, and some proprietary derivatives of phenol.

*Weak aqueous phenol solution* (0.5 to 1.0%) has a lower surface tension than water and may be used for washing and dressing in emergencies, but carbolic dressings should never be left in contact with skin, mucous membrane or wounds for more than thirty minutes to an hour unless a *very* dilute solution is used, since, like bichloride, they will cause coagulation of the live tissues (gangrene) which may be followed by serious infection. *Aqueous 1 to 2 per cent crude carbolic solutions* (also *cresol* and similar compounds) make excellent disinfectants for household use, as well as in the barn.

*Crude cresols* form colloidal (milky) suspensions in water and are therefore specially effective because each colloidal droplet consists of *concentrated* cresol. This is true of many colloidal disinfectants. Their uses are similar to those of phenol and they are more effective and less expensive. There are many excellent cresol preparations on the market. They have a clinging odor. Most of them contain a surface tension reducent. Good examples are *Lysol*,

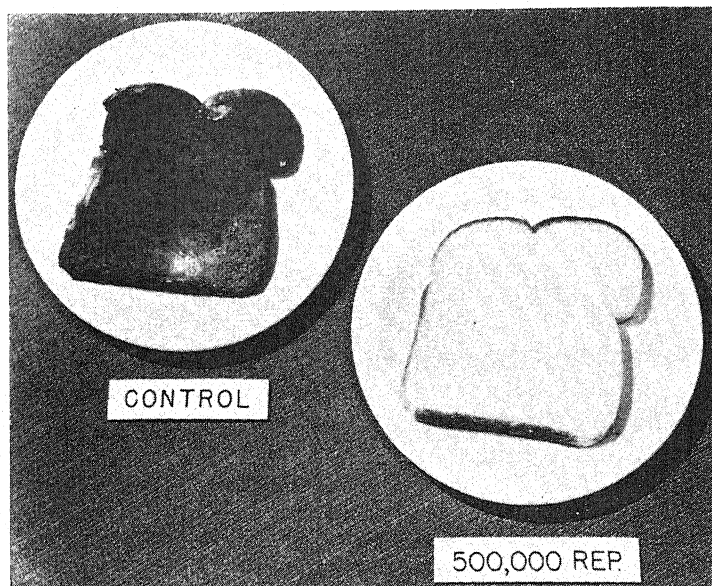


Fig. 19-4. Efficacy of electron irradiation in food preservation. The control slice of bread was not irradiated. After three months of incubation it is almost completely decomposed and blackened by growth of molds, bacteria and related microorganisms. The irradiated slice remains "fresh." Cereals, grains, and other foods can be freed from weevils, trichina worms and other pathogenic and/or damaging parasites by proper irradiation. (Photo courtesy of General Electric, X-ray Department.)

and saponated cresol solution, U.S.P. The preparation of such solutions is an important industry.

6. **Radiations.** Ultraviolet light is actively microbicidal when applied properly. It has very slight powers of penetration. Bacteria hidden behind a particle of dust may escape it. It cannot penetrate glass. Exposure must be *direct* and *sufficiently prolonged*. Eyes must be protected from it, as it can severely damage them.

Cathode rays (electron beams) are expensive to apply but *can* be developed on a commercial scale. They can be made to penetrate thin metal, paper or plastic sheets and have been used successfully to sterilize packaged meats, vegetables and other foods (Fig. 19-4). (See also Chapt. 43.)

7. **Alcohols.** Several alcohols have been found to have some value as disinfectants, but only two are in general use; these are ethyl and isopropyl alcohols. Like the phenol derivatives and quaternary compounds, disinfectant value is closely related to chemical structure. Isopropyl alcohol is used as "rubbing alcohol," purchasable in any drug store. As disinfectants, these alcohols are of about equal value and usefulness. They have the disadvantage of other coagulative substances, in that they are inactivated by mucus, pus, etc. Tests in pure cultures of bacteria show these alcohols (70% volumetric) to be quite effective against a wide variety of non-sporeforming bacteria. In the presence of excessive extraneous coagulable material, they cannot be relied on for strong disinfectant action. They are effective for disinfecting *properly*

wiped clinical thermometers. As a hand wash they are commonly recommended in 70% solution.

As an adjuvant, ethyl alcohol is useful because it increases the wetting properties of aqueous solutions of other disinfectants. In some instances, as in certain of the quaternaries, it greatly enhances their apparent effectiveness. It is frequently used as a hand wash, with small amounts of green soap, perfumes, etc., or other disinfectants, mixed in.

8. **Quaternaries.** These compounds have been discussed in detail in the section on surface tension and wetness. The value of these substances as microbicidal agents is still in question. They are readily inactivated by competitive adsorption with soap, by hard water, by acids and alkalies, and by organic matter. They are more effective in warm than in cool water. Anionic substances precipitate with cationic substances, and each with  $H^+$  or  $OH^-$ , respectively. There seems no doubt that many quaternaries exert bacteriostatic action in dilutions as high as 1:120,000. Probably they are bactericidal in greater concentrations. As commonly used, they do not kill spores. In this they resemble nearly all other chemical disinfectants except concentrated acids and alkalies and ethylene oxide. In aqueous solutions they appear to be of limited value in dealing with higher fungi or with tubercle bacilli. Tinctures (solution in alcohol) appear to be highly effective, especially against tubercle bacilli.

**Ethylene Oxide.** The use of gases for disinfection and control of disease by fumigation has its origin in very early history. The use of glycol vapors to disinfect *air* has been the subject of much recent investigation. In 1949 Phillips and Kaye proposed the use of gaseous *ethylene oxide* for disinfection of surgical instruments, bedding, and other objects. Ethylene oxide has the formula  $CH_2-CH_2$  and is liquid at temperatures below  $10.8^\circ C$ . ( $51.4^\circ F.$ ), its boiling



point. It is inexpensive and may be obtained in small cylinders. It is somewhat poisonous and highly inflammable, but this property may be diminished by diluting it with 90%  $CO_2$ . A commercial mixture of this sort, called *Carboxide*, is available. However, even this form of ethylene oxide must be handled carefully. Special autoclaves are available in which carboxide may be used instead of steam. Pressures range around 20 pounds. This is especially valuable for plastic, rubber and leather goods, and delicate instruments which would be ruined by heat and steam.

It is possible to sterilize fluid culture media by mixing fluid ethylene oxide with them. The oxide disappears on warming, leaving no apparent trace except an increase in acidity. It apparently kills spores and viruses.

#### REFERENCES

- Adelberg, E. A.: The use of metabolically blocked organisms for the analysis of biosynthetic pathways. *Bact. Rev.*, 1953, 17:253.
- Bellamy, W. D., and Lawton, E. J.: Problems in using high-voltage electrons for sterilization. *Nucleonics*, 1954, 12:54.
- Brochure. Chilean Iodine Educational Bureau, Inc. Iodine Abstracts and Reviews, 1955, 3:No. 2.
- Brochure. Wescodyne: The All-purpose Iodine Disinfectant for Hospital Use. West Disinfecting Co., Long Island City, N. Y.



- Butterfield, C. T., Wattie, E., and Chambers, C. W.: Bacterial efficiency of quaternary ammonium compounds. *Pub. Health Rep'ts*, 1950, 65:1039.
- Frobisher, M., Jr., Sommermeyer, L., and Blackwell, M. J.: Studies on disinfection of clinical thermometers. *Appl. Micr.*, 1953, 1:187.
- Gershenfeld, L., and Witlin, B.: Iodine as a sanitizing agent for food and eating utensils. *Am. J. Pharm.*, 1951, 123:87.
- Joint Committee on Atomic Energy, 84th U. S. Congress. Radiation Sterilization of Foods, May 9, 1955. U. S. Government Printing Office, Washington, D. C.
- Judge, L. F., Jr., and Pelczar, M. J., Jr.: The sterilization of carbohydrates with liquid ethylene oxide for microbiological fermentation tests. *Appl. Micr.*, 1955, 3:292.
- Klarman, E. G.: The role of antagonisms in the evaluation of antiseptics. *Ann. New York Acad. Med.*, 1950, 53:123.
- Klarman, E. G., and Wright, E. S.: Are "quats" fungicidal? *Am. J. Pharm.*, 1954, 126:267.
- Klarman, E. G., and Wright, E. S.: Are quaternary ammonium compounds sporicidal? *Am. J. Pharm.*, 1950, 122:330.
- Migaki, H., and McCulloch, E. C.: Survivor curves of bacteria exposed to surface active agents. *J. Bact.*, 1949, 58:161.
- Miller, O. T., Schmitt, R. F., and Phillips, G. B.: Applications of germicidal ultraviolet in infectious disease laboratories. *Am. J. Pub. Health*, 1955, 45:1420.
- Oetlingen, W. F.: Phenol and its derivatives. *N. I. H. Bul. No. 190*, 1949.
- Sommermeyer, L., and Frobisher, M., Jr.: Laboratory studies on disinfection of rectal thermometers. *Nursing Research*, 1953, 2:85.
- Stewart, J. A., and Clark, B. S. (Pilcher, R. W., Ed.): *The Canned Food Reference Manual*. Am. Can Co., New York, 1947.
- Van Eseltine, W. P., and Hucker, G. J.: The effect of organic matter on germicidal action of the quaternary ammonium compounds. *New York State Agr. Exp. Station Tech. Bul.*, 282, 1948.
- Vera, H. D.: Sterility testing: The control of efficiency of sterilization techniques. *Appl. Micr.*, 1953, 1:117.

## Destruction, Removal and Inhibition of Microorganisms

### 3. ANTIBIOTICS

FOR DECADES microbiologists have known that certain air-borne, antagonistic, saprophytic microorganisms got into their cultures as contaminants and suppressed the growth of the species which they desired to cultivate. The suppression of desired organisms was, of course, due to antimicrobial substances (antibiotics) given off by the saprophytes (Fig. 20-1). But the phenomenon was so commonplace and attention was so fixed on other problems, that it was pushed aside as merely an inevitable nuisance. The true significance of the observation was overlooked until Fleming (afterwards Sir Alexander Fleming, Nobel Prize Winner) in 1929 appreciated its possibilities and acted upon the basis of his idea (Fig. 20-2).

#### PENICILLIN

The air-borne, antagonistic, saprophytic contaminant which first attracted Fleming's attention was the common mold, *Penicillium notatum*. It occurred to Fleming to experiment with its antimicrobial action. By passing broth cultures of the mold through filters he removed the mold filaments. Thus he was able to study the activity of the growth products alone as they occurred in the broth. He found that the clarified, sterile broth contained a highly potent, antimicrobial principle, the activity of which was readily demonstrated in contact with sensitive microorganisms. He called this principle *penicillin*. In a much purified and refined form it is the penicillin used therapeutically today.

Fleming realized the practical possibility of his discovery but was not in a position to develop it more fully and for some years it remained relatively unknown. Attention was called to it again in 1935 but still it awakened little interest.

Realizing that many pathogenic organisms are rapidly destroyed in the soil, Waksman (Nobel prize winner) suggested that the search for an effective antagonistic microorganism be carried to the soil. In 1939 Dubos found, in

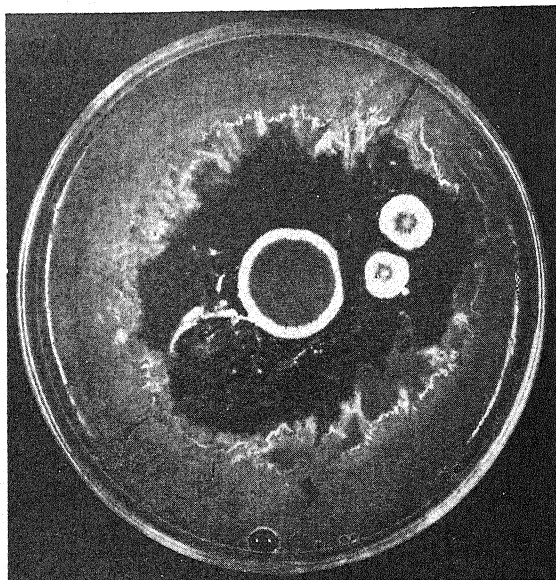


Fig. 20-1. Three large colonies of *Penicillium notatum* grown on a plate of agar heavily inoculated with a penicillin-sensitive organism. The dark zone around the three colonies, covering about one third of the total area of the plate, represents bacterial inhibition. This is the phenomenon noted by Fleming in 1929 which led to his discovery of penicillin. (Merck & Co., The Story of Penicillin, 1944.)

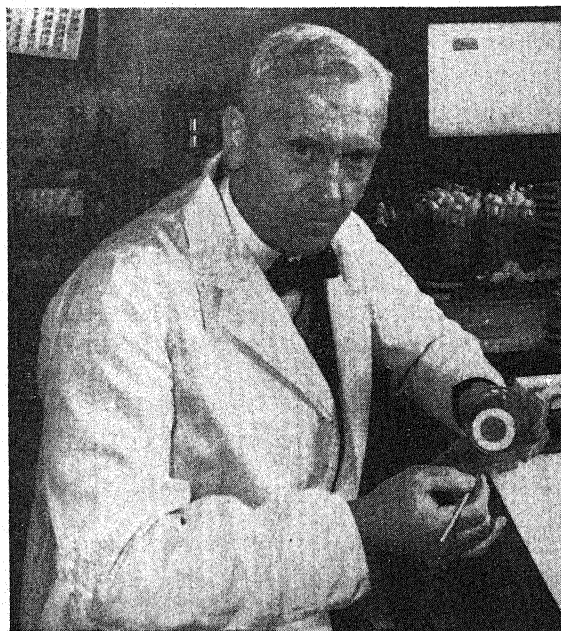


Fig. 20-2. Prof. Sir Alexander Fleming, discoverer of penicillin; Nobel Prize winner. Dr. Fleming is pointing with his inoculating needle at a giant colony of *Penicillium notatum* on an agar plate. (From Pfizer Spectrum, appearing in the Journal of the American Medical Association.)

bogs, organisms (*Bacillus brevis*) which produced valuable antibacterial substances (*gramicidin*, *tyrothricin*, etc.). But these, while of enormous value therapeutically and of great interest scientifically, were too toxic for internal use. Dubos's discoveries reawakened interest in Fleming's observations on *Penicillium notatum* and work on penicillin was begun in England on a large scale by Florey, Chain, Abraham, and others (the Oxford group) in 1940.

Since that time penicillin has played a role in the most titanic war in human history, in the most prodigious piece of cooperative research ever organized (prior to atomic research); in a great industrial development; in the most complete control over a variety of diseases ever achieved by man in a short time; and, because of its impact on venereal diseases, in a tremendous new social and moral trend, the possibilities of which are still only partly realized. The military importance of penicillin in World War II, then just beginning, can hardly be estimated; but the importance of American facilities for mass production of penicillin, then not available in Europe, was soon of great benefit to all concerned.

**Production.** Production of penicillin involves cultivation of *Penicillium notatum* under conditions most favorable to growth and penicillin production, i.e., at around 24° C. Penicillin production is best at a pH between 7 and 8. As the mold is strictly aerobic, exposure to air is essential.

Commercially, submerged growth in vigorously aerated tanks holding thousands of gallons of medium (Fig. 20-3) is most widely used today.

In all methods suitable medium is inoculated with spore suspensions.

During the 7 to 14 days of incubation in the large vat of aerated, fluid medium the mold excretes at least three waste substances which are of importance: (a) the yellow pigment *chrysogenin*, which must be removed by adsorption with charcoal; (b) *penicillin*; (c) *notatin* or *penicillin B* (this occurs especially if the acidity of the medium is too great). This is removed during the final purification process.

**MEDIUM.** Different formulae are doubtless in use by various manufacturers but basically they are similar and are mainly modifications of the following:

Corn-steep liquor*	40	ml
Lactose monohydrate	27.5	gm
NaNO <sub>3</sub>	3.0	gm
MgSO <sub>4</sub> · H <sub>2</sub> O	0.25	gm
KH <sub>2</sub> PO <sub>4</sub>	0.50	gm
ZnSO <sub>4</sub> · H <sub>2</sub> O	0.044	gm
MnSO <sub>4</sub> · H <sub>2</sub> O	0.020	gm
Glucose monohydrate	3.0	gm
Water ad.	1000	ml

After about 7 days, growth is complete and pH rises to 8.0 or above and penicillin production ceases.

When no more penicillin is being formed, the masses of mold growth are separated from the culture fluid by centrifugalization and filtration and the process of extracting the penicillin from the clear fluid begins. This is quite

\* Corn-steep liquor is a by-product of the distilling industry, being the water used to soak (steep) the corn prior to fermentation. It contains various growth factors (vitamins), proteins, carbohydrates, etc., and is one of the best sources of nutriment for penicillin production by many strains of the mold.

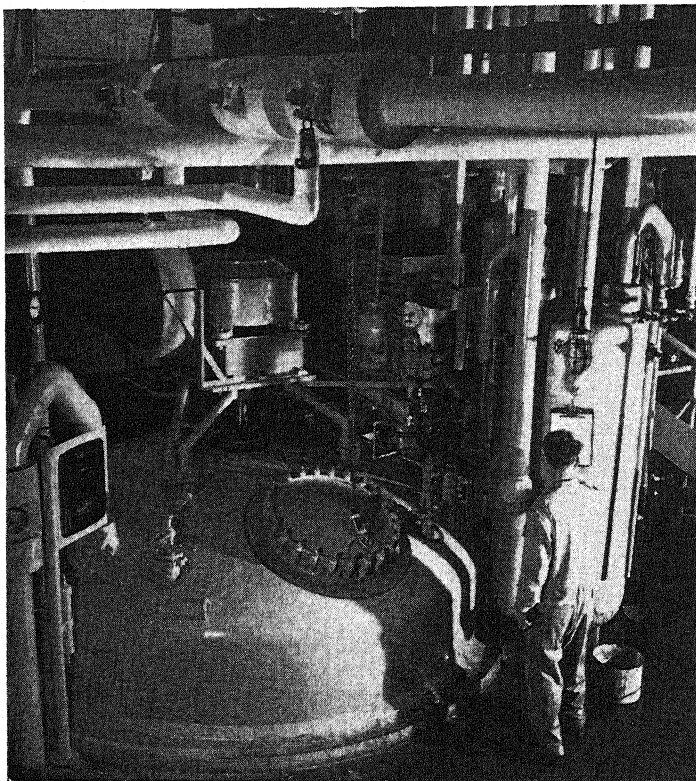


Fig. 20-3. Like a steel igloo, the top of a 9,000-gallon aerated-growth tank rises above the floor level of the penicillin plant at Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y. Like an iceberg, nine tenths of the huge fermentation tank is out of sight below the floor. Small tank at the right contains chemicals to prevent excessive foaming of the liquid containing the mold. (Peter Winkler, Lederle Laboratories, Inc.)

complex and involves various extractions with organic solvents, crystallizations, etc.

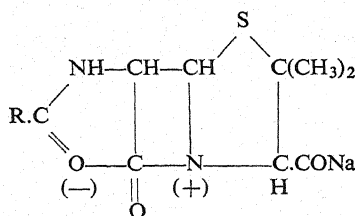
**Properties.** The "pure" substance (as will be shown later, in reality a mixture of related penicillins called X, G, F, dihydro F, flavicidin and K) occurs as an acid. Penicillin is readily soluble in water and alcohol but the latter inactivates it. It is quickly destroyed by acid. In the crystalline form it is quite stable, even to several days at 100° C and for years or more refrigerated in the dark.

Among the most valuable properties of penicillin is its relatively low toxicity for man and animals even in large doses, whereas its potency is such that a concentration of 0.000001 gm per ml will exert marked bacteriostatic effects. It may be administered subcutaneously, intravenously or locally. Although the acidity of gastric juice destroys it to some extent, as well as certain bacteria in the intestines, and although it is not so well absorbed, much penicillin is given orally because of the convenience. About five times the intramuscular dose must be given. Buffers are often added to offset gastric acidity.

**Mode of Action.** Penicillin probably acts as a specific cell poison (for certain species only), probably by the inactivation of one or more components of their respiratory enzyme system in a manner analogous to sulfonamide drugs. It appears to act primarily as a bactericidal agent; to a less extent bacteriostatically. Much depends on the species of organism involved and the concentration of penicillin.

Any chemotherapeutic agent which acts bacteriostatically in the body gives the normal defense mechanisms of the body (leukocytes, etc.) the upper hand in the struggle against invading microorganisms. This will be more fully clarified farther on.

**Chemistry and Varieties of Penicillin.** The penicillins are strong, monobasic acids, pH about 2.8. The general chemical structure provisionally assigned to them is:



The R varies in the different penicillins as follows:

RELATIONSHIPS OF NATURAL PENICILLINS

PENICILLIN	SIDE CHAIN (R)
F or I	n-pentenyl: $\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}=\text{CH}\cdot\text{CH}_2-$
G or II	benzyl: $\text{C}_6\text{H}_5\cdot\text{CH}_2-$
X or III	p-hydroxy-benzyl: $\text{HO}\cdot\text{C}_6\text{H}_4\cdot\text{CH}_2-$
K or IV	n-heptyl: $\text{CH}_3(\text{CH}_2)_6-$
Dihydro F	$\text{CH}_3(\text{CH}_2)_4-$
Flavicipidin	$\text{CH}_3\text{CH}=\text{CH}(\text{CH}_2)_2$

Laboratory synthesis of penicillin was announced shortly after the formula was known. The substance actually produced was penicillin G. A number of other substances closely related, which also had antibiotic properties, were produced experimentally during the research, thus revealing the possibility that *better artificial penicillins and other antibiotics* may be made than are available from natural sources.

**Biosynthesis of Penicillins.** In addition to artificially synthesizing penicillin, it has been found possible to induce *Penicillium* cultures to synthesize new forms of penicillin. The method used involves two main steps: (a) to "split" the penicillin molecule chemically into its constituent radicles; (b) to "feed" to the cultures of *Penicillium notatum* these various parts of the penicillin molecule, or *chemically related* substitutes. By extra feedings of the parts of the normal penicillin molecule (called *precursors*), the production of normal penicillins has been greatly increased. By feeding substances chemically related to normal precursors, but of slightly different molecular structure, new

and more valuable penicillins have been synthesized by the mold, using these abnormal precursors.

This same sort of thinking has been applied to other processes in which a wanted product is synthesized by a living organism. The field of *biosynthesis* is clearly wide open for the ingenious, the well-trained and the energetic student.

**Various Strains of *Penicillium Notatum*.** Fleming's strain of *P. notatum* for a time was thought to be unique. It was later found that some "wild" strains of this organism were better producers of penicillin, and that other, closely related organisms, especially *P. chrysogenum*, also produce penicillin. These findings led to a very extensive search for better strains of *P. notatum* and for penicillin-producing strains of other organisms. Many thousands of cultures were tested and it was found that the penicillin-producing property is widely distributed in the *P. notatum-chrysogenum* group and that some strains are better than others, especially for the various methods of production, i.e., some were good in tanks, not so good on surfaces, and vice versa.

Later, naturally-occurring variants of the best strains were tested and it was found that strains might be of two types: a conidial or C type, producing relatively little mycelium, many conidia and much penicillin; and an M or mycelial type, producing much mycelial growth, but relatively few conidia and little penicillin. Much care is, therefore, exercised to maintain cultures in the C condition and to save stocks of spores of these strains for "seed." The search for a "super" strain continues. Irradiation with ultraviolet light has furnished many thousands of mutant strains for testing. The method has yielded some very potent penicillin-producing mutants. It is especially interesting to note the usefulness of this method in inducing the development of variants which produce large amounts of one or another of the various penicillin isomers, F, G, X or K.

**Factors Inhibiting Penicillin.** **PENICILLINASE.** Many microorganisms produce an enzyme, *penicillinase*, which rapidly destroys penicillin. In the presence of such species the action of penicillin is suppressed or eliminated. Among the species producing penicillinase are *Escherichia coli*, members of the genus *Bacillus* and some species of *Shigella*. Many species which do not produce penicillinase are, nevertheless, resistant to penicillin for other, mostly obscure, reasons.

**Development of Resistance or Drug-Fastness; Dependence.** An undesirable aspect of the continued clinical use of antibiotics (as well as of sulfa drugs) is the emergence of variant strains: (a) having *increased resistance* to the drugs (drug-fast strains); or (b) wholly *dependent* on the drugs. Drug-fastness and drug dependence have been described in Chapter 18.

**Laboratory Uses of Penicillin.** The value of penicillin as a selective bacteriostatic agent in laboratory bacteriology was recognized by Fleming and others. It is a convenient aid to the isolation of penicillin-resistant organisms (such as the whooping cough and influenza bacilli) from throat cultures since it suppresses the growth of unwanted, penicillin-sensitive organisms. It is also used for suppressing bacterial growth in studies of viruses and rickettsiae and pleuropneumonia organisms, the latter three groups of microorganisms being almost wholly resistant to most antibiotics (see Chapters 5 and 13).

**The clinical uses of penicillin** are beyond the scope of this discussion, but

some of the common infectious organisms against which it is more or less effective are listed here.

*Highly susceptible*

*B. anthracis*

Various clostridia including *Cl. tetani* and *Cl. welchii*

*D. pneumoniae*

*Neisseria* spp., including gonococci and meningococci

Micrococci

*Streptococcus pyogenes*, etc. (Fig. 20-4)

*C. diphtheriae* (in vitro)

*Moderately susceptible*

*Borrelia* of relapsing fever

Various alpha ("viridans") type hemolytic streptococci

*Leptospira* spp.

*Treponema pallidum*

Note that all of these except *Neisseria* are gram-positive species.

It is generally ineffective in salmonellosis and shigellosis (typhoid fever and bacillary dysentery), influenza, pertussis, tularemia, brucellosis, and other infections caused by gram-negative bacilli, as well as viral, rickettsial, and PPLO infections and infections with protozoa, yeasts, and molds.

**Standardization.** The Oxford or Florey Unit of penicillin is the least amount necessary to inhibit completely the growth of a certain strain of *Micrococcus pyogenes*, var. *aureus* in 50 ml of a standardized, meat-extract broth.

In 1944 an international unit was defined as 0.6 microgram of an arbitrary International Standard Sample, this quantity having been found to equal 1 Oxford unit.

**Methods of Assay and Sensitivity Testing.** Five types of procedure are in



Fig. 20-4. Child with severe infection of tissues of the lower face and chin and around eyes, due probably to hemolytic streptococci. At left, at beginning of treatment with penicillin; center, 96 hours later; right, 9 days after beginning treatment. (Herrell, Proc. Staff Meet., Mayo Clinic, vol. 18.)



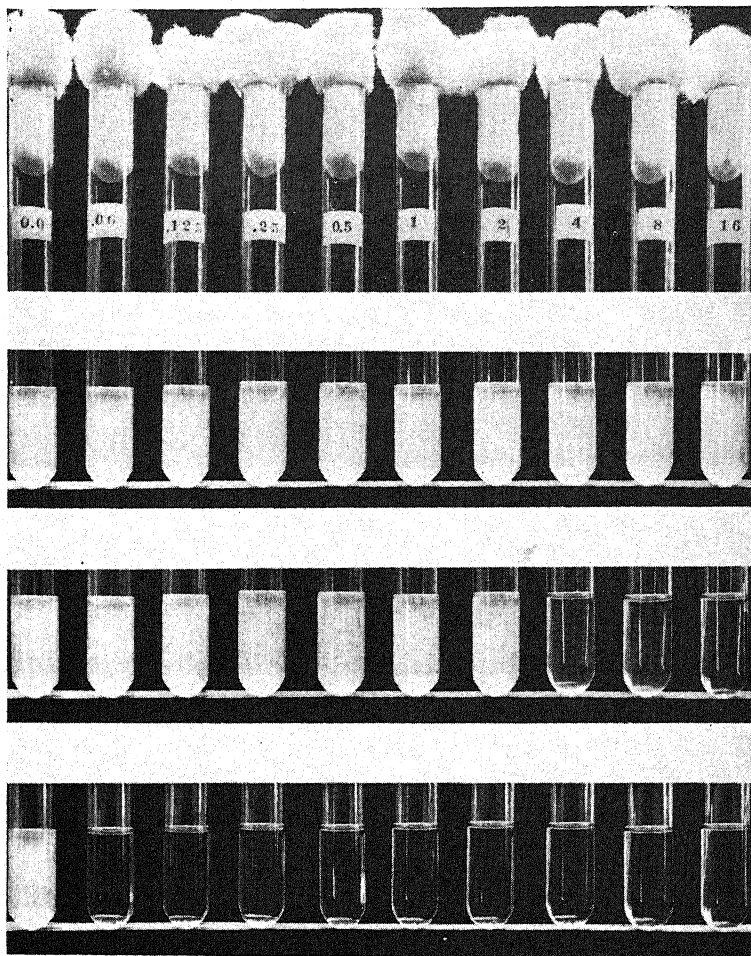


Fig. 20-5. Testing sensitivity of a bacterium to antibiotics or other chemotherapeutic agents by the "tube dilution" method. The top row of tubes of broth culture contain "Agent A" in increasing amounts as shown by the figures on the tubes. The second row contains "Agent B" in the same amounts while the third row contains "Agent C" in the same dilutions. All tubes were inoculated with "Organism X" from the same culture at the same time and incubated together. Luxuriant growth, as shown by white turbidity, has occurred even in the highest concentration of Agent A, showing that Organism X (perhaps from a patient very ill with this infection) is not in the least affected by this drug. The drug of choice will be Agent C which prevents growth of Organism X even in the smallest concentration. Agent B is slightly effective. The tube at the left of each row is a control tube containing no inhibitory agent. (Courtesy of The Abbott Laboratories, North Chicago, Ill.)

use for assaying the potency of, or sensitivity of microorganisms to, penicillin and other antibiotic preparations: (1) the serial dilution method in broth; and (2) the serial dilution method in agar (agar-streak method); (3) the cylinder-plate method; (4) the disk or tablet method; (5) the blood-plate method. These methods, modified, are also used for measuring sensitivity of "un-

known" organisms to standardized, graded amounts of antibiotics and sulfonamides. This is commonly done in hospital laboratories.

There are sources of error in all of these methods unless used and interpreted by experts.

1. THE SERIAL DILUTION ASSAY METHOD IN BROTH. Serial dilutions of the "unknown" antibiotic sample are made in uniform amounts of standard broth in culture tubes. These are then inoculated with a uniform amount of the test organism, which is selected on the basis of its known degree of sensitivity. Turbidity (or its absence) is noted and may be measured by means of a photoelectric turbidimeter. The turbidities (amounts of growth) are compared with a dilution-series made in exactly the same way but with a "known" antibiotic reference standard.

The method is relatively laborious and requires sterilization of the sample, which destroys some of its potency. However, it is one of the most accurate procedures and results can be stated in exact terms of units of antibiotic.

This same procedure, reversed, is often used to measure the sensitivity of an "unknown" organism, isolated from an infected patient, to various antibiotics. Graded, known, strengths of antibiotics are placed in series of tubes.

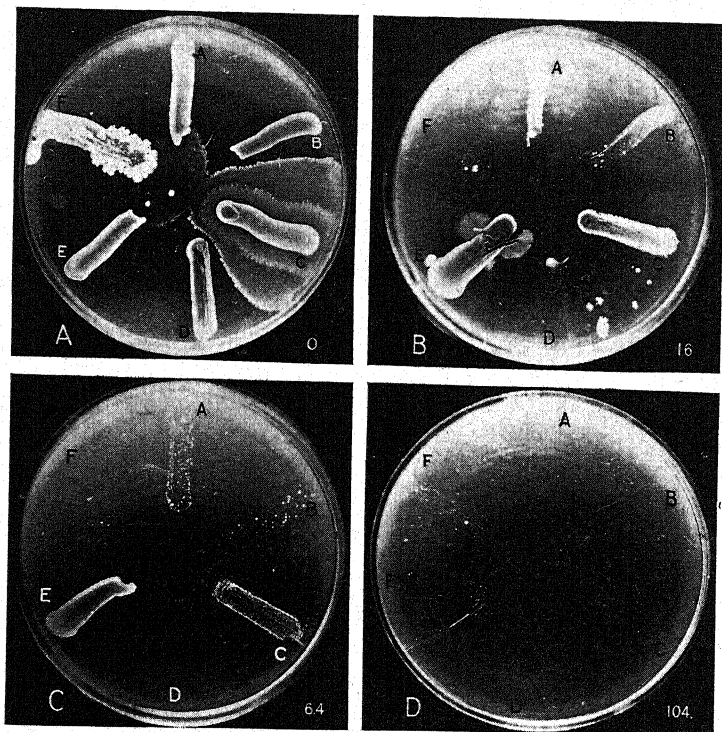


Fig. 20-6. Antibacterial activity of streptomycin. A, *Escherichia coli*. B, *Salmonella typhi*. C, *Proteus* sp. D, *Klebsiella pneumoniae*. E, *Pseudomonas aeruginosa*. F, *Mycobacterium tuberculosis*. The letters refer to streaks of growth on the plates. The numerals indicate micrograms of streptomycin per ml. of agar. Note the great sensitivity of *Mycobacterium tuberculosis* (F) to the drug. Streptomycin is particularly valuable in the treatment of tuberculosis. (Courtesy of Merck & Co., Inc.)

Each tube is inoculated with a drop of the organism whose sensitivity is being tested. If growth occurs in all tubes, the organism is resistant; if no growth occurs, the organism is sensitive. Growth in some of the tubes permits a measure of the sensitivity of the organism (Fig. 20-5).

2. SERIAL DILUTION (STREAK ASSAY) METHODS IN AGAR. These are rapid, do not require a sterile antibiotic sample, permit the use of several test organisms at once, and can be used to test substances not dissolved in water. The method is much used for preliminary assays of crude material.

Graded dilutions of the substance to be tested are placed in a series of Petri dishes. Into these are poured about 10 ml of melted and cooled agar which is thoroughly mixed with the antibiotic dilution by tilting the plates back and forth. After the agar has hardened, the plates are marked into several sectors, on each of which is streaked a different test organism. After incubation, the highest dilution of the antibiotic inhibitory to each organism is recorded (see Fig. 20-6).

This method, reversed, also is often used for sensitivity testing by using *known* strengths of antibiotics and *unknown* organisms.

3. THE CYLINDER PLATE METHOD. This is one of the earliest procedures used for antibiotics assay. A series of steel, glass or porcelain cylinders about 10 by 6 mm in dimensions, with perfectly square-cut ends, like a drainage tile, are sealed, by touching them, while slightly heated, to the upper surface of already inoculated, solid agar in a Petri plate. The solutions of antibiotic to be tested are placed in the cylinders (Fig. 20-7). The plate is then incubated. Diffusing into the agar, the antibiotic solution reveals its potency by the width of the zone of inhibition of growth in the agar around the cylinder. The test organism, agar, density, time, temperature, pH, etc., must be very exactly standardized. This is true in *all* of the methods described here.

4. THE DISK OR TABLET METHOD. This method is less exact than the procedures just described but is quick, convenient and economical. It is therefore a preferred and widely used method for many purposes not requiring high degrees of accuracy. The principle is the same as the cylinder plate method. The cylinders are replaced by disks or tablets. This method has been adapted to: (1) assaying the potency of new preparations of antibiotics; (2) determining the amount of antibiotic in body fluids of patients; (3) testing the resistance or susceptibility of strains of bacteria to antibiotics.

In disk and tablet procedures nutrient agar, of a composition appropriate to the organism being tested, is heavily inoculated with the desired organism. The inoculum may be streaked all over the surface of the solidified agar or mixed with the agar while still fluid before pouring the plate. If an antibiotic solution of unknown potency is being assayed (e.g. manufacturer's sample, blood, urine, etc.) the organism used is a stock strain of *known sensitivity* to *standard doses* of that antibiotic.

The antibiotic solution to be tested is applied to the inoculated agar in measured quantities in disks of sterile filter paper or in small "tablets," which are placed on the surface of the agar plate before incubating. The antibiotic diffuses outward from the disk or tablet and inhibits growth in the agar around it. The width of the zone of inhibition is a resultant of: (a) the concentration of antibiotic in the solution; (b) the sensitivity of the organism

used; (c) ability of the drug to diffuse into the agar; (d) time and temperature of incubation; pH and composition of the medium, etc.

The converse of this procedure is seen in the common practice of testing the antibiotic resistance of "unknown" strains of bacteria causing infections in patients. In such tests the "unknown" organism, instead of a stock strain, is used to inoculate the agar. Various disks with several antibiotics in *known*, graded dosages are placed on the agar. After incubation some of the disks will have growth in contact with their edges, showing that they have exercised no inhibition (the organisms are completely resistant). Other disks will be surrounded by clear zones varying in width (Fig. 20-8). The presence or absence of a zone of inhibition, rather than its width, will guide selection of the antibiotic to be used in the patient. Appropriate disks, with measured amounts of all commonly available antibiotics and sulfonamide drugs, are available commercially.

5. BLOOD AGAR PLATE METHOD FOR RAPID SENSITIVITY TESTING. Sterile blood agar is poured into a Petri dish to a depth of about 2 mm (base layer).

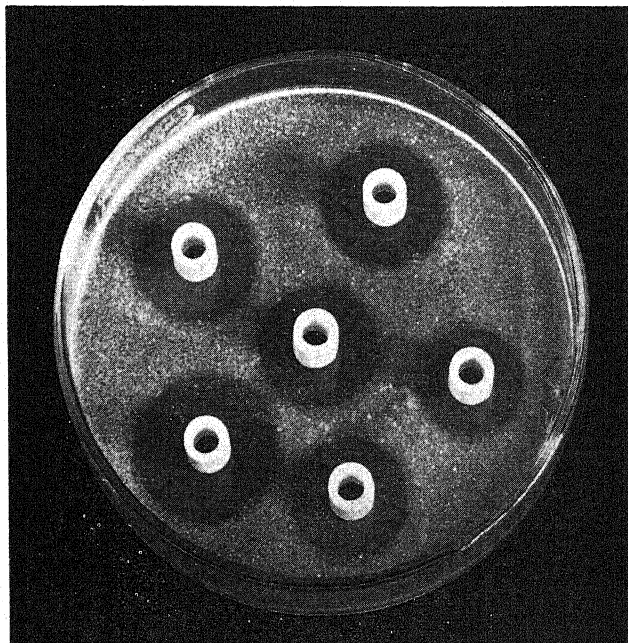


Fig. 20-7. Cylinder plate standardization of penicillin. The culture plate contains a very carefully standardized medium, uniformly and heavily seeded with staphylococci or spores of *B. subtilis*. In each cylinder was placed a measured quantity of penicillin-containing extract. Following incubation under standard conditions, growth of bacterial colonies gave the medium the pebbled, gray appearance seen everywhere except around the cylinders. Here, growth has been inhibited by penicillin. Measurement of these zones of inhibition permits standardization of penicillin in Oxford units by comparison with zones produced under identical conditions of test by standard solutions of known unit strength. The sensitivity of an unknown organism to any antibiotic is tested in exactly the same way; inoculation of the plate is made with the "unknown" in place of the staphylococci or *B. subtilis* routinely used. Known amounts of various antibiotics may be placed in the cylinders. (Therapeutic Notes, March, 1944, Parke, Davis & Company.)

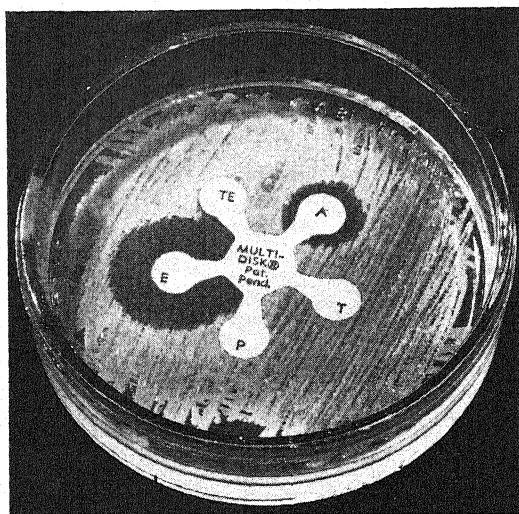


Fig. 20-8. A simplified procedure for the disk method of determining sensitivity of an organism to antibiotics or other drugs. The plate was prepared by inoculating the surface of the agar with the organism to be tested. The starshaped piece of filter paper was then placed on the surface and the plate was incubated. The disk at the tip of each arm of the star contains a different drug. Obviously drug *E* is most effective in inhibiting the organism, drug *A* much less so, while the organism is wholly resistant to drugs *P*, *T*, and *TE*. Many other combinations of drugs in similar arrangements are available. (Photo courtesy of The Abbott Laboratories, North Chicago, Ill.)

Similar fluid agar is heavily inoculated with the organism being tested. After thoroughly mixing, this is poured over the base layer to an equal depth (seed layer). When solid, antibiotic disks are placed on the surface and the plate is placed in the incubator. The plate may be examined as early as two hours and every hour thereafter. Diffuse growth of the organism, whether actual colonies are visible or not, produces a marked discoloration and/or hemolysis of the blood *except* where inhibited under and around disks containing effective antibiotic concentrations. Here the blood remains unchanged.

#### ANTIBIOTICS FROM STREPTOMYCES

*Streptomyces* (see Chapter 30) are bacteria which have some of the structural and biochemical properties of molds, like *Penicillium notatum*. Streptomycetes are, however, minute in size as compared with true molds and have other characters relating them to the Schizomycetes.

A number of species of *Streptomyces* have been found to produce antibiotics of medical and commercial value. Important among these are streptomycin; chlortetracycline (*Aureomycin*); oxytetracycline (*Terramycin*); tetracycline (*Achromycin*); erythromycin (*Ilotycin*, *Erythrocin*); carbomycin (*Magnamycin*); and chloramphenicol (*Chloromycetin*). (See Table 15.) There are others, and more are constantly being introduced. Some of these are said to be active against certain of the large viruses, others against certain viral neoplasms, some against certain protozoa and so on. A description of streptomycin will

exemplify all of the group, although each has its own special properties and values.

**Streptomycin.** The discovery of streptomycin dates only from 1944 and followed naturally in a long series of researches by Waksman and his collaborators (Fig. 20-9) into the numbers and kinds of antagonistic microorganisms in the soil. The source organism produces conidia and aerial mycelia and is one of the order Actinomycetales: *Streptomyces griseus*, whence the name streptomycin.

The principles underlying production, purification, assay and standardi-

**Table 15.** *Partial List of Antibiotics.\**

ANTIBIOTICS		CHARACTERISTICS†	SOURCE
COMMON NAME	TRADE NAME		
Penicillin‡		Gram-positive bacteria; <i>Treponema</i> ; <i>Neisseria</i>	<i>Penicillium notatum</i>
Fumagillin		<i>Entamoeba histolytica</i>	<i>Aspergillus fumigatus</i>
Streptomycin		<i>Myco. tuberculosis</i> ; Gram-negative bacteria	<i>Streptomyces griseus</i>
Dihydrostreptomycin		Like streptomycin	Streptomycin
Tetracycline	Achromycin	Broad-spectrum	Chlortetracycline
Oxytetracycline	Terramycin	Broad-spectrum	<i>Streptomyces rimosus</i>
Chlortetracycline	Aureomycin	Broad-spectrum	<i>Streptomyces aureofaciens</i>
Chloramphenicol	Chloromycetin	Broad-spectrum	<i>Streptomyces venezuelae</i>
Erythromycin	Ilotycin, Erythrocin	Broad-spectrum (not Enterobacteriaceae)	<i>Streptomyces erythreus</i>
Carbomycin	Magnamycin	Like erythromycin	<i>Streptomyces halstedii</i>
Neomycin B‡	Flavomycin	Mycobacteria	<i>Streptomyces fradiae</i>
Viomycin	Viocin	Like penicillin	<i>Str. floridae</i> ; <i>Str. funiceus</i>
Chrysomycin		Bacteriophage	<i>Streptomyces</i> sp.
Oligomycin		Fungi	<i>Str. diastatochromogenes</i>
Netropsin		Some viruses	<i>Streptomyces netropsis</i>
Anisomycin		Protozoa	<i>Streptomyces</i> sp.
Nystatin		Fungi	<i>Streptomyces noursei</i>
Cycloheximide	Actidione	Saprophytic fungi	<i>Streptomyces griseus</i>
Bacitracin		Like penicillin	<i>Bacillus licheniformis</i> (?)
Polymyxin B‡		Gram-positive bacteria	<i>Bacillus polymyxa</i>
Xerosin		Some viruses	<i>Achromobacter xerosis</i>
Pyocyanin		Miscellaneous	<i>Pseudomonas aeruginosa</i>

\* Several not listed here are valuable commercially, agriculturally, horticulturally, and so on.

† Not necessarily the only activity.

‡ Several of these antibiotics are in reality mixtures consisting of related compounds like the penicillins, polymyxin A, B, C, D, etc., carbomycin A and B and so on.

|| Not used medicinally. One of the first known antibiotic substances.





Fig. 20-9. Dr. Selman A. Waksman. (Courtesy Society of American Bacteriologists.)

zation of streptomycin are analogous to those pertaining to penicillin, differing mainly in respect to cultural details and technical procedures appropriate to the organism and substance involved.

Aeration of the culture is essential for rapid streptomycin production, which is at its maximum in two to three days. Various strains of *Streptomyces griseus* are more or less active producers of the drug.

The unit of streptomycin is analogous to the unit of penicillin. The S unit, originally proposed, is the least amount which, when present in 1 ml of nutrient medium, just inhibits a standard strain of *Escherichia coli*. A new unit is based on crystalline material, of which 1 gamma (1 microgram: 0.000001 gm) is equal to 1 unit (1 Oxford unit of penicillin = 0.6 gamma).

For detection of streptomycin, the streak-plate method serves as a preliminary determination. More accurate definitions of potency depend on the cylinder-plate method, using a certain strain of *Micrococcus pyogenes*, var. *aureus*, or the disk method. A broth dilution method has also been utilized. The drug may be inactivated with cysteine or by semicarbazide.

The formula of streptomycin is  $C_{21}H_{39}O_{12}N_7$ . The drug is much more stable than penicillin, both in dry form and in solution. In the refrigerator the solutions retain potency for months, and at 37° C for about two weeks.

Boiling for 10 minutes produces about 50 per cent loss of potency. Streptomycin in vitro is bactericidal in high concentrations; bacteriostatic in low concentrations.

**SCOPE OF ACTION.** Its scope of action is in some respects broader than that of penicillin, being active against a variety of gram-negative and gram-positive organisms, some of which are listed below.

Gram-positive	Gram-negative
<i>Mycobacterium tuberculosis</i>	<i>Aerogenes</i> species
<i>Micrococcus pyogenes</i>	<i>Salmonella typhi</i>
<i>Diplococcus pneumoniae</i>	<i>Pasteurella tularensis</i>
<i>Streptococcus</i> , species	<i>Klebsiella</i> species
<i>Bacillus subtilis</i> (and related species)	<i>Brucella abortus</i>
<i>Erysipelothrix</i>	<i>Proteus vulgaris</i>
Not:	<i>Salmonella paratyphi B</i>
Clostridia	<i>Hemophilus pertussis</i>
	<i>Hemophilus influenzae</i>
	<i>Pseudomonas aeruginosa</i>
	<i>Escherichia</i> species

It has been used in tuberculosis with striking yet disappointing results. The disappointment resulted only because too much was expected. It is a valuable drug in this disease when properly used.

Streptomycin has been used effectively in many infections with gram-negative rods not susceptible to penicillin or sulfonamides. It is not so rapidly destroyed in, or excreted from, the body as penicillin. Some toxic effects (vertigo, deafness, etc.), have been described. It must be used only under medical supervision.

**THE MODE OF ACTION OF STREPTOMYCIN** is not entirely clear. Like penicillin, its effect appears to depend on blocking certain phases of *aerobic* respiration or synthesis. Both streptomycin and dihydrostreptomycin are much less active anaerobically than aerobically. Both are mainly bactericidal in their action, rather than bacteriostatic.

No enzyme such as penicillinase has been reported, but resistance (drug-fastness) of organisms may be induced and, unfortunately, often occurs in tubercle bacilli in patients during treatment. Strains of tubercle bacilli wholly dependent on streptomycin are also found in treated patients.

**Chloramphenicol.** The organism producing chloramphenicol is much like other species of *Streptomyces*. It was found in 1947 by Burkholder in soil collected near Caracas, Venezuela, and is called *S. venezuelae*. The drug is a by-product of growth quite analogous to streptomycin, but it is inhibitory to a much wider range of organisms (except tubercle bacilli). It may be given either by mouth or intravenously.

It affects many species of gram-positive and gram-negative bacteria in high dilutions. Unlike either penicillin or streptomycin, it is effective against the large viruses, such as those of lymphogranuloma, "parrot fever" (psittacosis) and smallpox; and also against rickettsiae, such as those causing Q fever, the typhus fevers, and Rocky Mountain spotted fever. It is the first antibiotic discovered to have definite viricidal and rickettsicidal properties. It



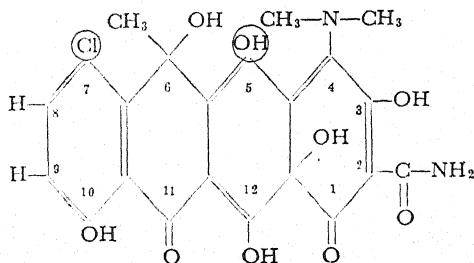


Fig. 20-10. Tetracycline group formula. For explanation see text.

has proven of inestimable value in the treatment of many infectious diseases. Because of its wide range (spectrum) of activity, including bacteria, viruses and rickettsiae, it is classed as a *broad-spectrum* antibiotic. It is bacteriostatic in action.

The therapeutic excellence of chloramphenicol led to intensive chemical studies, as in the case of penicillin. The formula was soon learned, and the drug synthesized (Chloromycetin) in 1949 in the laboratories of Parke, Davis and Company.

Chloramphenicol must be used only under medical supervision.

**The Tetracyclines.** This group includes three antibiotics: tetracycline (*Achromycin*), chlortetracycline (*Aureomycin*) and oxytetracycline (*Terramycin*). These have similar molecular structures based on tetracycline which is shown in Figure 20-10.

Chlortetracycline differs in having a chlorine atom attached to carbon atom No. 7 (encircled). Oxytetracycline has an hydroxyl group attached to carbon atom No. 5 (encircled). All have similar antibiotic properties. They are bacteriostatic in effect. They are effective against many gram-negative and gram-positive species of bacteria, some viruses and some rickettsiae. For this reason, along with chloramphenicol, erythromycin and carbomycin, they are called "broad spectrum" antibiotics and have similar ranges of therapeutic activity. However, in any given patient, any one of these drugs may at times show surprising irregularities and give unexpectedly brilliant results or fail. They are *not* identical.

#### ANTIBIOTICS FROM THE GENUS *BACILLUS*

Several useful antibiotics have been derived from gram-positive, aerobic, spore-forming bacteria closely related to, or identical with, *Bacillus subtilis*. One of these antibiotics is bacitracin; discovered by F. L. Meleney.

**Bacitracin** was first described in 1945. The source organism is a particular strain of *Bacillus subtilis*, a common and harmless organism widely distributed in dust. It was found contaminating a wound in a patient named Tracy; hence baci-tracin. Bacitracin is a yellowish powder readily soluble in water. Like penicillin it is active clinically mainly against infections due to gram-positive bacteria. However, it also affects the gram-negative meningococcus (cause of meningitis), gonococcus (cause of gonorrhea) and *Hemophilus influenzae*, as well as *Treponema pallidum* (cause of syphilis). Unlike penicillin, streptomycin, chloramphenicol and the tetracyclines, it is best used only for topical applications, although it has been used internally in pneumonia.

Bacitracin is representative of a number of polypeptide antibiotics with similar properties, derived from other species of the genus *Bacillus*. Among these are polymyxin B (*B. polymyxa*); bacillomycin (*B. subtilis*); biocerin (*B. cereus*); and circulin (*B. circulans*). They are mainly bactericidal in action.

#### NON-MEDICAL USES OF ANTIBIOTICS

**Industrial Uses.** In Chapter 44 some industrial uses of antibiotics are discussed. Here we need only point out that some of the antibiotics not suitable for medical use (as well as useful drugs) are finding uses as preventives of microbial spoilage of industrial commodities: paper, leather, etc. The use of chlortetracycline as a preservative of dressed poultry for retail was approved by Federal authorities on November 30, 1955. Doubtless other foods will be preserved with antibiotics. Some antibiotics are useful in research and special work of various sorts such as isolation of viruses and rickettsiae (see Chapter 5). Several are very useful in controlling diseases of commercially valuable plants: fruit trees, crops, and so on. Medicinal uses are only one aspect of the field of antibiotics.

**Antibiotics as Growth Stimulants.** One of the most interesting and unexpected developments in connection with antibiotics has been the discovery that a number of them greatly enhance the rate of growth and well-being of chickens, pigs, turkeys, and rats. They are of no benefit to ruminants because of the difference in anatomy and physiology. The tetracyclines, penicillin, streptomycin and chloramphenicol, all exhibit this property. They are being used in commercial stock feeds.

The exact mechanism of growth stimulation is not clear. It might be due to suppression of growth of bacterial parasites in the intestinal tract, but this is not finally proven. The usual dosage is below such inhibitory levels.

An undesirable result of feeding antibiotics to stock is the development of drug-fast strains of bacteria capable of producing drug-resistant infections of man and animals, thus offsetting the beneficial effects of feeding the drugs to farm animals. However, difficulties arising from this source appear to be rare.

In 1952 it was observed that certain antibiotics have a marked stimulating effect on the growth of plants and plant tissues. The importance of this to agriculture, horticulture, etc., can scarcely be estimated. Such studies should stimulate the graduate student in the fields of botany and zoology as well as in microbiology.

**Antibiotics for Plant Diseases.** The stock and poultry raiser is not the only beneficiary of the research on antibiotics. Bacterial and fungal diseases of plants which, as recently as 1956, bid fair to wipe out pear growing, chrysanthemum propagation and other valuable plant industries, now yield to antibiotics. Streptomycin, the tetracyclines, chloramphenicol and others, but especially the first because of its low cost, are being marketed to control the microbial pests. Fireblight, a devastating bacterial scourge of pomaceous fruits (pears, apples, etc.), is controlled with sprays of streptomycin and oxytetracycline. Bacterial blight of beans can be controlled very well by spraying with streptomycin solution. Other antibiotics (helixin, griseofulvin, etc.) of no medicinal value, are very effective in such diseases as gray mold of lettuce seedlings, early blight of tomatoes, and so on. The antibiotics are in some

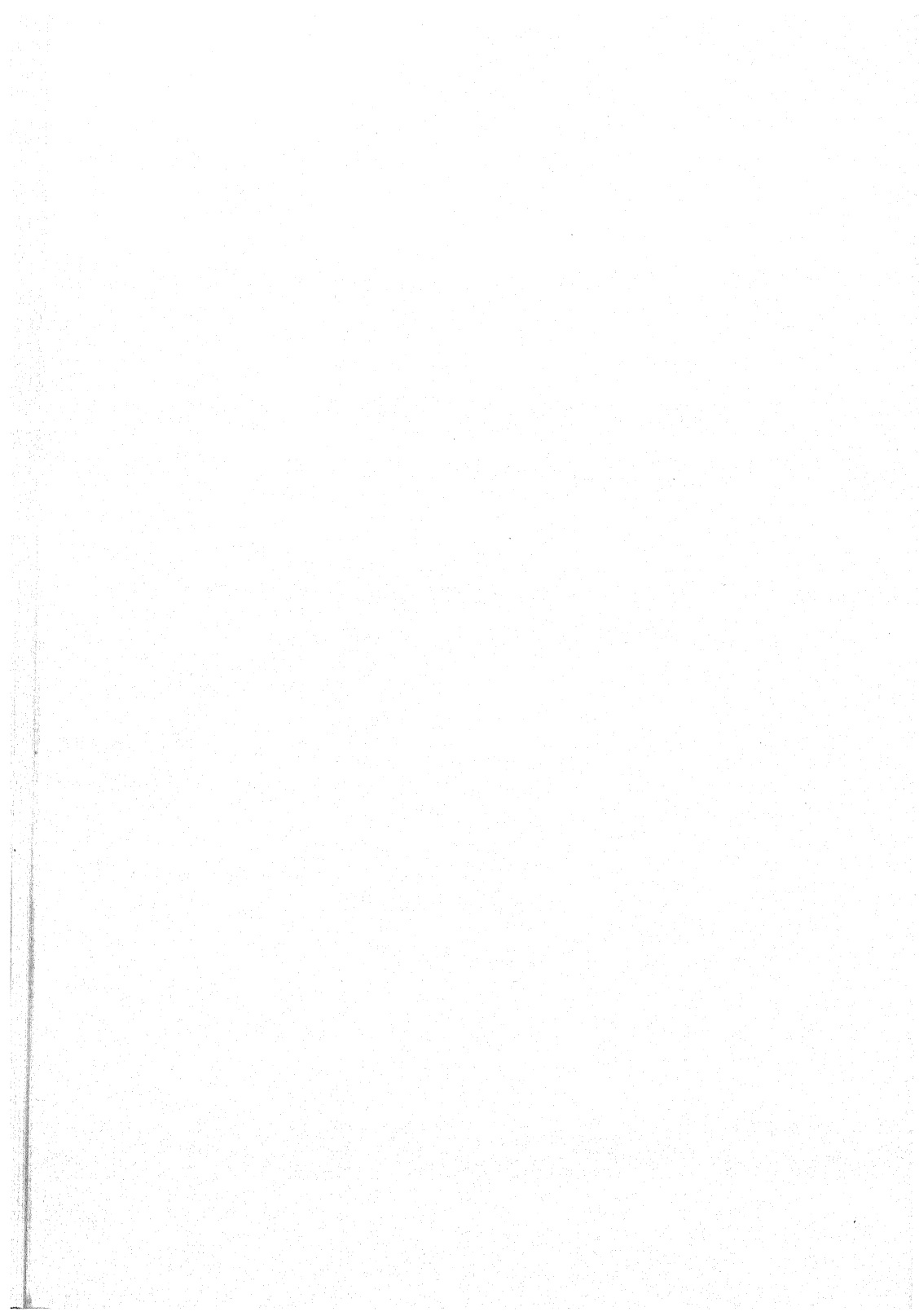
cases applied to the surface of plants as sprays; in others, cuttings are dipped into antibiotic solutions; in others the roots are immersed.

Results differ, depending on rate of penetration of the antibiotic into the plant (which in turn depends on the kind of antibiotic and species of plant), the microorganism involved, and other factors. It is puzzling to find that some antibiotics having no action against certain microorganisms when tested *in vitro*, are very effective against those same microorganisms in certain plants.

#### REFERENCES

- Alcorn, S. W., and Ark, P. A.: Movement of certain antibiotics in cuttings of *Pyracantha* and carnation. *Appl. Micro.*, 1956, 4:126.
- Anonymous: A rapid method for the determination of microbial susceptibility to antibiotics. Dept. of Microbiology. U.S.A.F. School of Aviation Medicine, Randolph Field, Texas, 1955.
- Arnstein, H. R. V., and Grant, T. P.: The metabolism of the penicillia in relation to penicillin biosynthesis. *Bact. Rev.*, 1956, 20:133.
- Boyd, J. W., Bluhm, H. M., Muirhead, C. R., and Tarr, H. L. A.: Use of antibiotics for the preservation of fish and sea foods. *Am. J. Pub. Health*, 1956, 46:1531.
- Collier, H. O. J.: Chemotherapy of Infections. John Wiley and Sons, New York, 1954.
- Collins, A. M., Craig, G., Zaiman, E., and Roy, T. E.: A comparison between disk-plate and tube-dilution methods for antibiotic sensitivity testing of bacteria. *Can. J. Pub. Health*, 1954, 45:430.
- Durbin, C. G.: Antibiotics in food preservation. *Am. J. Pub. Health*, 1956, 46:1306.
- Eagle, H., and Saz, A. K.: Antibiotics. *Ann. Rev. Micro.*, 1955, 9:173.
- Eagle, H., Levy, M., and Fleischman, R.: The effect of the pH of the medium on the antibacterial action of Penicillin, Streptomycin, Chloramphenicol, Terramycin, and Bacitracin. *Antibi. and Chemoth.*, 1952, 2:563.
- Editorial: Antibiotics against neoplasms. *Brit. Med. J.*, 1954, Feb. 6.
- Editorial: Antibiotics and growth. *Brit. Med. J.*, 1954, Sept. 11, 635.
- Editorial: Chlortetracycline, a food preservative. *J.A.M.A.*, 1956, 160:779.
- Editorial: Modern antibacterial agents. *J.A.M.A.*, 1955, 159:1458.
- Elek, S. D., and Hilson, G. R. F.: Combined agar diffusion and replica plating techniques in the study of antibacterial substances. *J. Clin. Path.*, 1954, 7:37.
- Gottlieb, D., Carter, H. E., Legator, M., and Gallicchio, V.: The biosynthesis of chloramphenicol. *J. Bact.*, 1954, 68:243.
- Grove, D. C., and Randall, W. A.: *Assay Methods of Antibiotics*. Medical Encyclopedia, Inc., New York, 1956.
- Hamilton, J. W., Szkolnik, W., and Sondheimer, E.: Systemic control of cherry leaf spot fungus by foliar sprays of acidione derivatives. *Science*, 1956, 123:1175.
- Hartman, F. W., Horsfall, F. L., and Kidd, J. G.: *Dynamics of Virus and Rickettsial Infections*. Blakiston Division, McGraw-Hill Book Co., New York, 1954.
- Heilman, F. R.: Antibiotics. *Ann. Rev. Microbiol.*, 1953, 7:219.
- Karel, L., and Roach, E. S.: *A Dictionary of Antibiosis*. Columbia University Press, New York, 1951.
- Kirby, W. M. M.: Antibiotics. *Ann. Rev. Microbiol.*, 1952, 6:387.
- Ley, H. L., Jr., and Smadel, J. E.: Antibiotic therapy of rickettsial diseases. *Antibiotics and Chemoth.*, 1954, 4:792.
- Martin, G. J.: *Biological Antagonism*. The Blakiston Co., Philadelphia, 1953.
- Newton, B. A.: The properties and mode of action of the polymyxins. *Bact. Rev.*, 1956, 20:14.
- Odum, E. P.: *Fundamentals of Ecology*. W. B. Saunders Co., Philadelphia, 1953.
- Pramer, D.: Antibiotics against plant disease. *Sci. Am.*, 1955, 192:82.
- Schatten, W. E., and Parker, R. F.: Evaluation of agar dilution method for determination of sensitivity of bacteria to antibiotics. *Proc. Soc. Exp. Biol. and Med.*, 1953, 83:574.
- Spaulding, S. H., and Anderson, T. G.: Selection of antimicrobial agents by laboratory means. *J.A.M.A.*, 1951, 147:1336.
- Umbreit, W. W.: Mechanisms of antibacterial action. *Ann. Rev. Micro.*, 1954, 8:167.

- Waksman, S. A.: Associative and antagonistic effects of microorganisms. *Soil Sci.*, 1937, 43:51.
- Waksman, S. A., Editor: *Perspectives and Horizons in Microbiology*. Rutgers Univ. Press, New Brunswick, N. J., 1955.
- Waksman, S. A., and Lechevalier, H. A.: *Guide to the Classification and Identification of the Actinomycetes and Their Antibiotics*. Williams & Wilkins Co., Baltimore, Md., 1953.
- Welch, H., and Ibanz, F. M., Editors: *Antibiotics Annual, 1955-1956*. Medical Encyclopedia, Inc., New York, N. Y.
- Welch, H., et al.: *Principles and Practice of Antibiotic Therapy*. Medical Encyclopedia, Inc., New York, 1954.
- Wiseman, R. F., Sarles, W. B., Benton, D. A., Harper, A. E., and Elvehjem, C. A.: Effects of dietary antibiotics upon numbers and kinds of intestinal bacteria in chicks. *J. Bact.*, 1956, 72:723.
- Wyss, O., et al.: Symposium on the mode of action of antibiotics. *Bact. Rev.*, 1953, 17:17.

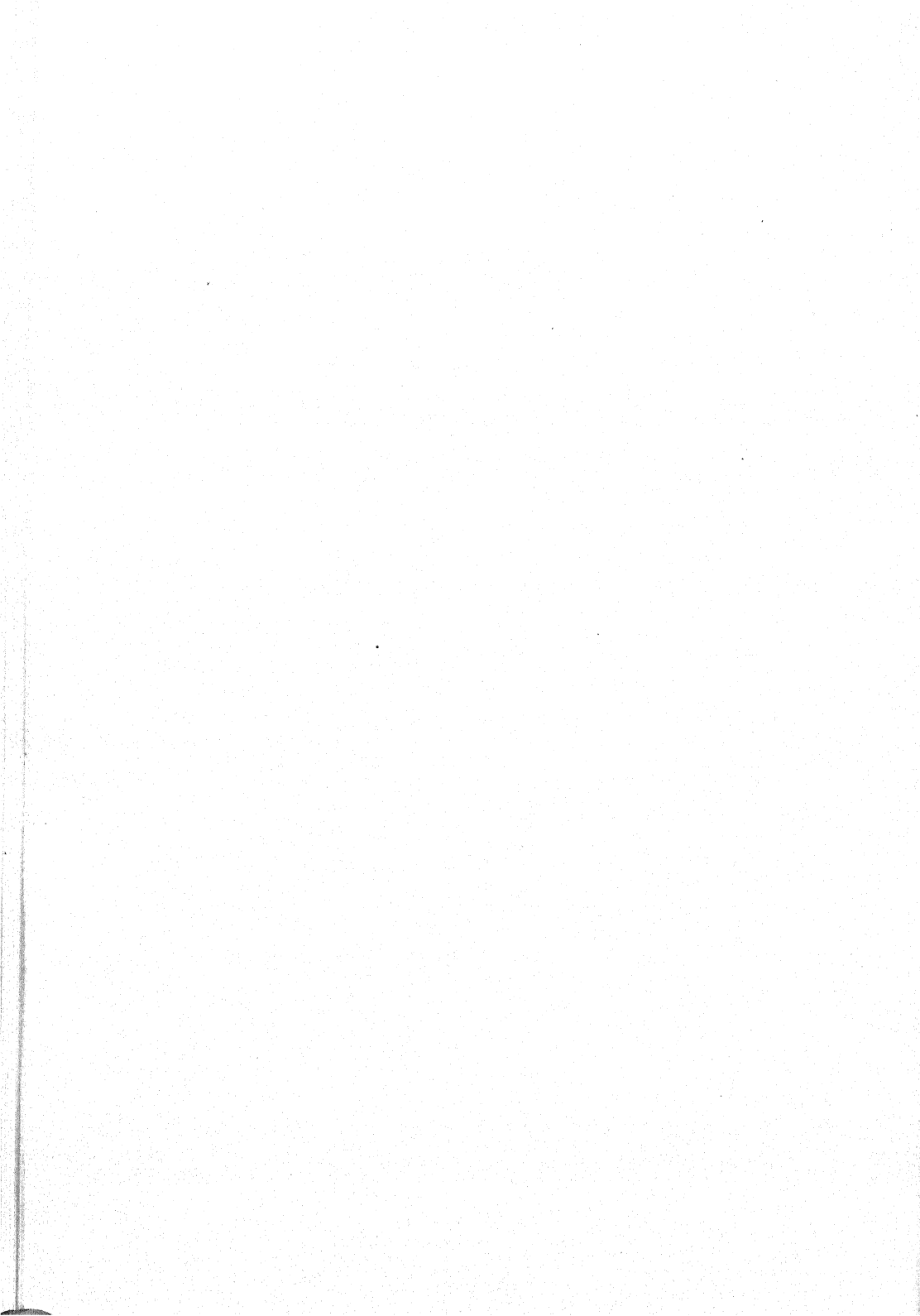


## SECTION 3

# *Immunology: Reaction of the Organism to Substances of Extraneous Origin*

THE TERM *Immunology*, used here largely because of tradition, and for convenience, was derived from studies on resistance to disease made more than a century ago. The modern concept of Immunology is that of a science having fundamental, biological significance which extends well beyond medical horizons. One might substitute for Immunology a phrase such as: Study of the reactions between tissue cells of living macroorganisms (especially vertebrates) and other organisms, especially (but not exclusively) microorganisms, living or dead, cellular or non-cellular, including parts and metabolic products thereof. This phrase is not only awkward but inadequate. Indeed, it would be difficult to define Immunology in a single sentence. One may more easily gain a true meaning of the term by reading this section.

Discussions of infection and disease are introduced in this section not only because they were originally the basis for the development of our knowledge of immunology, but also because they convey valuable practical information which will be useful to anyone as soon as he acquires it.



# Immunology and Microbiology

## 1. PRINCIPLES AND METHODS

IMMUNOLOGY DERIVES its name from an early concept that the reactions of animal tissue to substances extraneous to them were all mechanisms of defense against infectious disease, with little other use or significance. It is important for the student to realize that modern concepts of immunology embrace a much wider range of ideas, including many fundamental physical, chemical and physiological phenomena. Disease, and defense against it, are only a segment of modern immunology, though certainly a very considerable one. Further, modern methods of immunology are so widely used as laboratory tools in so many fields of work, and are based on such fundamental biological principles, that one must have a clear grasp of them before a detailed study of microbiology will prove profitable.

### BLOOD

Before discussing the nature of immunity, and methods for its study, it will be advisable to devote a little space to a description of blood and its constituents, since blood is a very important factor in immunological reactions and is much used in immunological investigations.

#### IMPORTANT BLOOD CONSTITUENTS

1. **Plasma.** For the purposes of this discussion, blood may be considered to have seven important constituent parts. First, there is the *plasma*; the yellowish, transparent, fluid part of the blood. It consists of a solution of proteins, salts, buffers, and other soluble substances including food for, and wastes from, the body cells. The plasma has in solution, also, the substances necessary to the formation of a *clot*.

2. **Fibrin Components.** The material responsible for clotting of blood is a complex, elastic protein called *fibrin*. Fibrin forms a microscopically fine, fibrous network in the blood after the blood leaves the body. This is seen as clotting. The fibrin meshwork soon shrinks to about half the original volume of the blood, squeezing out of its meshes the fluid portion of the blood, much as a wet, contractile sponge would exude water. Most of the blood cells (and bacteria if any are present) are caught and held in the fibrin clot (Fig. 21-1).



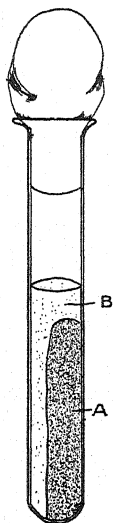


Fig. 21-1. Tube with clot (A) which has shrunk, enmeshing virtually all of the blood corpuscles and exuding clear serum (B).

3. **Platelets.** Associated with fibrin production are small bodies called *platelets*, or *thrombocytes* (*thrombus* is from a Greek root meaning clot). These are amorphous particles, variable in size, smaller than red corpuscles, deeply staining, which tend to form stellate groups. They may be cells or possibly fragments of marrow cells. Their role in coagulation of blood is important though not entirely clear. Platelets appear to facilitate clotting. The ability of blood to clot appears to be directly related to the number of platelets in the blood.

4. **Serum.** The fluid portion of blood, after it is separated from the fibrin, is called *serum*. Serum is essentially plasma minus the components of fibrin. Since fibrin usually enmeshes the blood corpuscles, the remaining serum is yellowish and transparent, like plasma. Serum contains most of the soluble substances, especially gamma globulins, in which the immunologist is interested. After blood has clotted the serum may be withdrawn in pipettes, centrifuged to remove stray red blood corpuscles, and stored in the refrigerator. It must be handled with every precaution to keep it sterile, for microorganisms gaining access to it would soon cause it to decompose. Immunologists often add minute quantities of preservatives.

5. **Lymph.** Lymph is very much like blood which has been deprived of red corpuscles by passing through the thin walls of the smaller blood vessels as through a fine filter. One sort of white blood corpuscles (*lymphocytes*) are found in it. The fluid thus seeping out of the vessels travels slowly in the fine spaces surrounding the blood vessels, and between the tissue cells and various organs. It eventually collects from all parts of the body in large drainage vessels (the lymphatic ducts) and is returned to the blood stream.

6. **Erythrocytes.** In the plasma, before clotting, are suspended the red corpuscles or erythrocytes (*erythro* is from the Greek word for red; *cyte* from the Greek word for cell). These are non-nucleated\* cells which give to blood

\* Mammalian erythrocytes are non-nucleated. In birds, amphibians and reptiles they are nucleated.

its opacity and red color and which carry oxygen from the lungs to the tissues. The color of erythrocytes is due to the red, oxygen-carrying substance, *hemoglobin*, which they contain.

Erythrocytes are about  $8\ \mu$  in diameter and number about 4 to 5 millions per cubic mm of blood (Fig. 21-2). When they are ruptured by plasmoptysis or other means, the hemoglobin is released and the blood becomes transparent, like red ink. The cells are said to have been "laked" or *hemolyzed*. Certain bacteria are very active in producing hemolysis by means of toxins. Some snake venoms are very hemolytic.

**7. Leukocytes.** In addition to erythrocytes, blood contains colorless, ameba-like cells called *leukocytes* (*leuko* is from the Greek word for white) which, in some cases, act as *phagocytes* (*phago* is from the Greek word for eat). Leukocytes are relatively large cells, 10 to  $20\ \mu$  in diameter, which have a definite nucleus and a means of locomotion like amebae. They normally number about 7 to 8 thousand per cubic mm of blood (Fig. 21-2). In many infectious processes, such as appendicitis, they greatly increase in numbers and the patient is said to have a *leukocytosis*. They act as scavengers and "policemen" in the blood.

Like amebae, leukocytes can ingest solid particles and, in the body, they serve by ingesting and destroying any small foreign particles such as bacteria, dead body cells, tissue detritus and the like, that may gain entrance to the tissues or blood stream (Fig. 21-3). The leukocytes, by their ameboid mobility, can leave the blood vessels and congregate in the tissues wherever any irritation exists.

## IMMUNOLOGY IN RELATION TO DISEASE

**Defensive Mechanisms.** The most important defensive mechanisms of the body against microorganisms and infectious disease may be divided into several categories as follows:

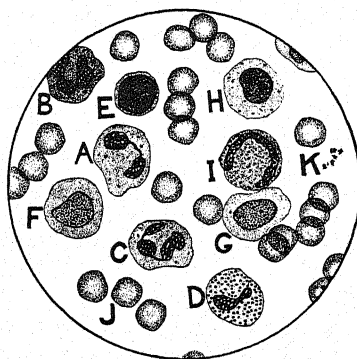
I. **Non-specific Resistance** (not directed against any specific disease but against infection in general).

A. *Genetic*

B. *Physiological*

1. State of general health, age, etc.
2. Mechanical and chemical
3. Phagocytosis

Fig. 21-2. Drawing of a smear of blood stained with Jenner's stain, showing common forms of blood cells. A, B, C, polymorphonuclear leukocytes with 2, 3, and 4-lobed nuclei, respectively. D, eosinophil, showing lobular nucleus and prominent, eosinophilic (red-staining) granules. E, lymphocyte. F, G, H, various forms of monocytes ("large lymphocytes"). I, lymphocyte with horseshoe-shaped nucleus ("transitional cell"). J, erythrocytes (red blood corpuscles); note biconcave-disk shape, thin at center. K, platelets. (Magnification about  $\times 1000$ .)



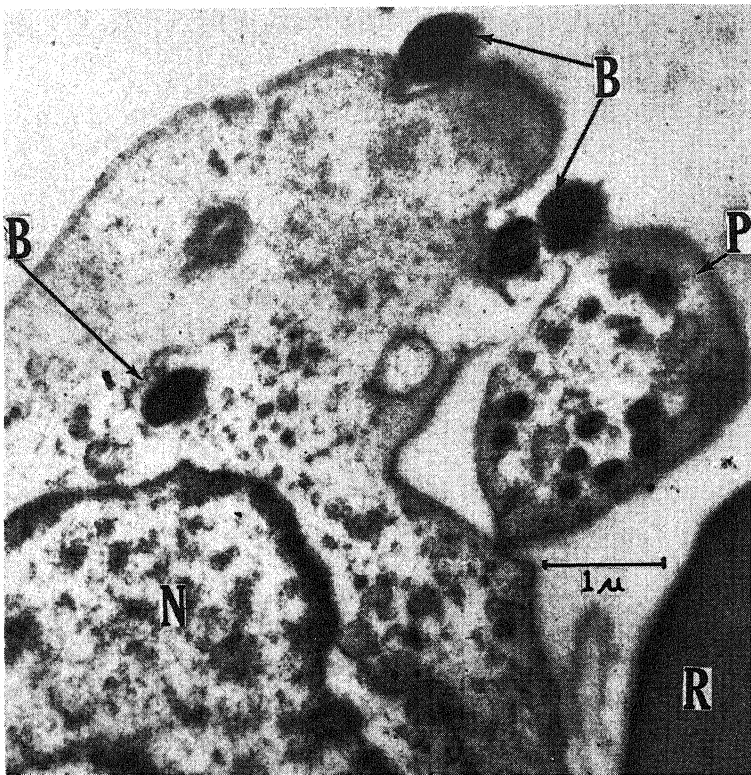


Fig. 21-3. Electronograph of an ultra-thin (around  $350\text{\AA}$ ) section of a part of a human polymorphonuclear leukocyte showing phagocytosis of virulent *Micrococcus pyogenes* var. *aureus*. *N* is part of one lobe of the nucleus of the leukocyte; note the well-defined nuclear membrane. *B* are cells of *M. pyogenes*; note that one bacterium is undergoing fission. The bacteria at the upper right are just being surrounded or grasped by the finger-like pseudopodia of the leukocyte. The bacteria will be "ingested" or drawn completely inside the leukocyte. They will be surrounded by a vacuole wall such as is seen to be forming around the bacterium at the left of center. Note the granular structure of the cytoplasm of the leukocyte and its differentiation from the nuclear plasm. *P* is a platelet; *R* is part of a red cell. Note the line indicating 1 micron. ( $\times 27,500$ .) (Courtesy of Drs. J. R. Goodman and R. E. Moore, V. A. Hospital, Long Beach, Calif.)

#### 4. Tissue immunity

#### 5. Properdin system

### II. Specific Resistance (developed by, and directed against, specific, infectious diseases).

#### A. Actively acquired

##### 1. Natural (due to recovery from infectious disease)

##### 2. Artificial (due to vaccination, injection of bacterins, etc., Chapter 22)

#### B. Passively acquired

##### 1. Natural (due to passage of maternal antibodies into fetus).

##### 2. Artificial (due to injection of antibody-containing serum).

#### C. Allergic (Chapter 23).

### I. NON-SPECIFIC RESISTANCE

**A. Genetic.** By this is meant *racial* or *species resistance* to disease, illustrated by the facts that horses do not have measles, man does not contract

fowl pox, etc. The reasons for this type of resistance are not always clear. They are probably quite complex, and differ in different species. For example, "cold-blooded" (*poikilothermic*) animals, and birds (normal temperatures around 104° F), are not susceptible to microorganisms which grow only at mammalian temperatures (normal temperatures around 98° F). However, as Pasteur proved in a very dramatic demonstration, chickens will die of anthrax (to which they are ordinarily resistant) if they are infected and then cooled by partial immersion in ice water.

**B. Physiological.** 1. STATE OF HEALTH AND AGE. Persons weakened by long overwork, starvation, exposure, age, disease, etc., are well known to become more susceptible than they are normally, to various infections such as tuberculosis, pneumonia, etc. Age is also an important factor. Children often suffer little from diseases fatal to older persons, and vice versa.

2. MECHANICAL AND CHEMICAL. These mechanisms are of a general nature, and offer protection against a variety of injurious agents.

*The outer skin*, especially of adults, is an obvious mechanical barrier to the entrance of foreign agents. It is chemically aided (against microorganisms) by its oily secretion and the acidity of perspiration. Acetic acid in perspiration is quite toxic to many bacteria. The skin may be by-passed by hypodermic needles, wounds, and by entrance of certain microorganisms via hair follicles and sweat glands.

*The hairs in the ears and nose* and the mucus on mucus-secreting membranes like those in eyes, nose and throat, mechanically entangle or enmesh particles of dust, insects, bacteria, etc. Secretions of *mucus* cause all of the respiratory and other surfaces, in contact with the exterior, to be sticky. Foreign bodies accumulate in the mucus. Removal to the exterior is then accomplished by sneezing, coughing, salivation, tears, etc. *The deeper air passages* are lined with *ciliated epithelial* (surface) cells. The cilia of these cells maintain a constant upward-waving movement which pushes mucus (with entrapped bacteria, dust, etc.) up to the larynx and throat where the mucus is either coughed up or swallowed. Further, these mucous surfaces are always "policed" by small numbers of leukocytes.

*In the gastrointestinal tract*, acidity of the stomach kills many swallowed microorganisms. *The upper intestine* is freed of microorganisms to a great extent by the *bile* and other *digestive juices*. The lower small intestine and large bowel contain great numbers of bacteria, many of which are highly pathogenic if they gain entrance to the blood or body tissues. They are normally held in check by the thick mucous membranes lining the intestines and by phagocytic and other mechanisms. A ruptured intestine, stomach or appendix is a source of serious infection which, untreated, is usually fatal.

*The adult genitourinary tract* is protected against most bacteria mainly by thick mucous membranes, leukocytes, and the flow of *urine*, which is normally acid.

3. PHAGOCYTOSIS. Centers of inflammation such as the bacteria in a boil, or a splinter of wood or other foreign particle, exert a strong attraction for leukocytes, especially polymorphonuclear leukocytes. These cells rapidly concentrate in such areas. The nature of this attraction is not fully known. It is called *chemotaxis* (*chemo* is from the Greek word for chemical; *taxis* is from the Greek word for orientation toward). The white cells try to dispose

of the foreign particles by ingesting them and also by giving off enzymes which will kill and destroy or digest them if possible. Often many leukocytes are in turn killed by poisons of the bacteria they ingest, and also by crowding and lack of oxygen in the region of concentration and activity.

The white, creamy material in a boil or other infected lesion, or around a "fester" splinter, is made up largely of dead and living white corpuscles, dead and living bacteria, tissue debris, lymph, fibrin and serum. It is called *pus* and the dead leukocytes in it are called *pus cells*.

**TYPES OF PHAGOCYTES.** Phagocytic cells are of two main types: fixed and wandering. The fixed phagocytes are found "built into" the linings of the blood vessels, especially in the liver, spleen and bone marrow. They are often called histiocytes (*histio* is from the Greek word for tissue). They take foreign particles from the blood as it streams by. They are part of what is called the *reticulo-endothelial system* of phagocytes.

The wandering phagocytes are the true white corpuscles or leukocytes, described above, which float *free* in the blood stream.

**PHAGOCYTOSIS A FIRST LINE OF DEFENSE.** The leukocytes and other phagocytic cells constitute an extremely important mechanism of defense against microorganisms and other foreign particles which may gain entrance to the blood and/or tissues.

**CELLULAR AND HUMORAL IMMUNITY.** The relation of phagocytosis to immunity was pointed out by Elie Metchnikoff about 1882 and was the origin of the doctrine of "cellular immunity." At about the same period specific, soluble proteins (globulins, notably *gamma globulins*), called *antibodies*, were discovered by Buchner, von Behring and others, in the serum of persons and animals who had been "vaccinated" or who had recovered from specific diseases. These antibodies were found to exert deleterious action against microorganisms. Antibodies were, therefore, hailed as the real basis of immunity to disease and gave rise to the doctrine of "humoral (fluid) immunity."

**PHAGOCYTES AND ANTIBODIES.** Long and intensive studies have shown that both antibodies and phagocytes are important in immunity. The antibodies, with one or two possible exceptions (antitoxins and cytolytic antibodies), appear to act mainly, if not exclusively, as *adjuvants* to phagocytosis.

Phagocytes are like a standing army; ready at all times for immediate duty. They are a first line of defense. Antibodies, on the contrary, are a physiological response to a stimulus and appear only hours, days or weeks *after* an infection has begun. Obviously they can be of only secondary (though usually critical) assistance.

**ANTIBODIES AND SURFACE PHAGOCYTOSIS.** The work of phagocytes is greatly facilitated by several factors, of which two important ones are: (a) *presence of antibodies*; (b) *presence of surfaces* against which phagocytes can hold or entrap bacteria in order to phagocytize them.

a. *Presence of Antibodies.* Many microorganisms, especially pathogenic species, have slimy surfaces which make it difficult for the leukocytes to grasp them with their pseudopodia preparatory to engulfing them. This is true especially if the microorganisms are floating freely in a fluid like blood. One may visualize the situation by trying to grasp wet watermelon seeds, especially when they are floating in water. Antibodies prepare the surfaces

of bacteria, especially those which are coated with slippery, slimy capsules, so that the bacteria are sticky and do not readily slip out of the grasp of the phagocytes. This preparation of the bacteria to be eaten more readily by the phagocytes is often referred to as opsonization (*opsonin* is from the Greek word meaning "to prepare food for").

b. *Presence of Surfaces.* If the phagocytes can get the bacteria "on the ropes," to use a prize-fighter's term, that is, against a *surface* where the organisms cannot back away, the phagocytes can grasp the bacteria much more effectively. The surfaces of other tissue cells or of any uneven or rough surface, or strands of fibrin, serve the purpose for the phagocytes very well. Thus, by means of *surface phagocytosis*, even in the absence of antibodies, the phagocytes can take up fully encapsulated bacteria very effectively. Phagocytosis is, therefore, not dependent on antibodies. Doubtless antibodies, if present, enhance the effectiveness of surface phagocytosis (Figs. 21-4 and 21-5).

4. **TISSUE IMMUNITY.** Infection under natural conditions brings the infecting microorganism immediately into contact with the living tissue cells of the host. Ordinarily these tissue cells, aided by phagocytes, localize and destroy the invader. The reaction is manifested as a local inflammation (defensive struggle), visible or imperceptible. This disappears with successful repulse of the invader.

But suppose the local tissues and phagocytes, while holding some, are unable to cope with *all* of the invaders. Some escape into the blood stream or lymph channels. "Blood poisoning" or *bacteremia* (bacteria in the blood stream) is present. Let us suppose that the phagocytes in the blood and tissues cannot immediately control these invaders either. The inflammatory reaction at the site of the initial infection, and wherever else the invading organisms may have localized, becomes more intense.

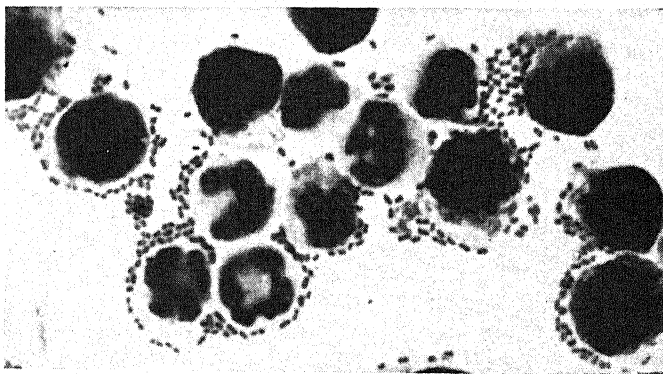


Fig. 21-4. Phagocytes surrounded by thousands of virulent pneumococci (*Diplococcus pneumoniae*), most frequent cause of lobar pneumonia. Note that *none* of the bacteria is *inside* of the phagocytes. The phagocytes have been unable to engulf the microorganisms because all are lying on a smooth, wet, slippery glass surface on which the phagocytes can get no "purchase" or foothold which will enable them to brace themselves to hold the pneumococci to ingest them. Compare with Figure 21-5. (Courtesy of Dr. W. B. Wood, Jr., and M. R. Smith, Washington University School of Medicine, St. Louis, Mo.)

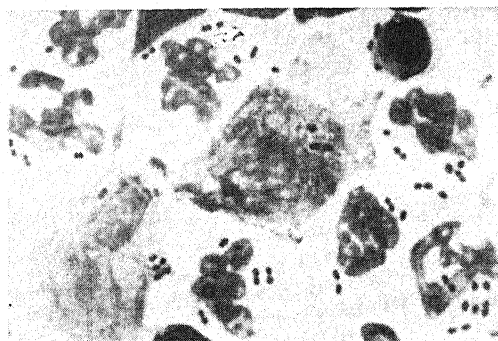


Fig. 21-5. A mixture of *D. pneumoniae* and phagocytes similar to that seen in Figure 21-4 except that in this situation there are tissue surfaces, angles, corners, fibrin shreds, irregularities and roughnesses where the phagocytes can trap and hold the slippery bacteria, enabling the phagocytes to ingest them. This phenomenon is called *surface phagocytosis*. Note that virtually *all* of the bacteria are *inside* of the phagocytes. (Courtesy of Dr. W. B. Wood, Jr., and M. R. Smith, Washington University School of Medicine, St. Louis, Mo.)

**LOCALIZING ACTION OF INFLAMMATORY TISSUE.** Now, the inflammatory tissue (or other reacting tissues) begins to form antibodies probably within a few minutes or hours after infection. It is probably due to this (in part at least) that inflammatory tissue has very marked power to *localize* and hold the invaders. Specific combinations occur between tissue cells and organisms. Increased phagocytosis of antibody-covered (opsonized) microorganisms, also occurs. If the local tissues, antibodies and phagocytes can then hold and destroy the invader, the infection is suppressed. If not, then the victim may succumb.

**5. THE PROPERDIN SYSTEM.** This is a group of serum components which, together, apparently play an important role in primary, non-specific resistance to infection. There are three components in the system: (a) *properdin*, a serum protein; (b) *magnesium* ions; (c) *complement*, a complex of serum proteins (C'1, C'2, C'3, C'4) which are very important in complementing the action of specific antibodies. Complement is discussed more fully later on.

Properdin itself is an enzyme-like agent. It appears to be closely related to, if not an actual part of, the complement complex. The amount of properdin in the blood seems to be directly related to the degree of non-specific resistance to numerous types of infection: bacterial, protozoal and viral. Injection of properdin-rich serum from animals having naturally high levels of properdin in their serum increases resistance to infection. Injection of certain carbohydrate complexes (zymosan) found in the cell walls of certain bacteria and yeasts greatly reduces properdin levels and, with them, resistance to infection. Electromagnetic irradiations and infection with certain bacteria also lower properdin levels.

In order for properdin to affect microorganisms it is necessary for  $Mg^{++}$  to be present. This is also true of complement. Complement, also, is necessary to the action of properdin. The exact mechanism of the action of the properdin system is not yet fully understood. Indeed, it seems to be so sadly true that the more we learn the less we understand. Shall we eventually know everything and understand nothing?

## II. SPECIFIC RESISTANCE

## A. Active Immunity

1. **Natural Active Immunity.** ANTIGENS. After recovery from many diseases such as Rocky Mountain spotted fever (rickettsial), measles (viral), diphtheria, whooping cough, typhoid fever (bacterial), the patient is so changed that he may never again, under ordinary circumstances, acquire the specific disease. He is said to possess natural, active immunity to the specified infection. This is because agents of infectious disease contain certain irritating foreign\* substances which stimulate the production of antibodies. Such antibody-generating substances are called *antigens*.

The irritation (or disease) caused by the antigens may be so mild (chronic or subacute or subclinical) as to pass unnoticed by the individual. On the other hand, the reaction may be rapid and violent (acute), with high fever. In any case, upon making proper tests, the patient's blood is found to contain protein substances called *antibodies*, *specific* for the antigens of the infecting microorganism. These antibodies neutralize the toxins or unfavorably affect the microorganisms, or both.

Often antibodies continue to be produced by the body cells long after the stimulus which excited their production disappears. It is in part to this that prolonged immunity, such as follows mumps, measles, and diphtheria, is due.

**Nature of Antigens.** In general, the most important antigenic substances are *soluble* polysaccharides and *soluble* proteins from any outside source, harmless or not: microorganisms, animal blood, egg, milk, serum or tissues, plant juices, etc.

All of these polysaccharide and protein substances act as *antigens*; i.e., all stimulate production of antibodies. They appear to enter into and/or irritate tissue cells which produce the *gamma globulins* of the blood plasma. The globulins produced in response to any given antigen almost exactly resemble that particular antigen structurally and chemically. These globulins are called *antibodies* because they tend to neutralize, inactivate, or destroy antigens.

**SPECIFICITY.** The structural resemblance between an antigen and the specific antibody which is produced to combat it is analogous to that between a lock and its specific key or between type and print.

Thus, the specially-designed antibody globulins can combine and react only with their own antigen and no other. A similar relationship is seen in that between an enzyme and its substrate. The relationship in both cases is spoken of as *specificity*.

The action of the antigen in impressing its physicochemical pattern on the

\* For the purposes of this discussion, the interior of the gastrointestinal tract is considered external to the body proper, as it is open to the outer world at both ends and has no direct connection with the principal body cavities or tissues. We shall, therefore, consider as *foreign* substances only those which get into the body *parenterally* (i.e., otherwise than through the gastrointestinal tract). This includes, generally, infecting micro- or macroorganisms, substances or bodies injected into the tissues with hypodermic needles, or introduced by means of splinters or wounds, or forced into the peritoneal (abdominal) or pleural (chest) cavity, or released or absorbed into the blood or lymph from mucus and other surfaces, or in any situation where they escape the digestive enzymes of the mouth, stomach or intestines.



globulin-forming cells is not fully understood. Since the globulin-forming cells die and are replaced, the successors presumably inherit the effects of the antigen. Or can it be that the antigen enters their genetic mechanism in somewhat the same relationship as prophage? Can infection, prophage, sexual fertilization, antigenicity, transduction, etc., all be different "variations on a theme?" These are merely hypothetical questions to stir up argument!

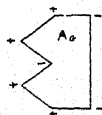
**NATURE OF SPECIFICITY.** Specificity (either in enzyme-substrate relationship or antigen-antibody relationship), is a matter of chemical and physical structure. For example, a "synthetic protein" antigen (of which several have been prepared experimentally by combining protein with certain inorganic radicles) having a given chemical structure (e.g.,  $\text{NH}_2\text{C}_6\text{H}_4 \cdot \text{AsO}_3\text{H}_2$  + protein) will, upon injection into the body, engender antibodies in the serum which react only with that compound. Let us alter the antigen, say by substituting a  $-\text{SO}_3\text{H}$  group in place of the  $-\text{AsO}_3\text{H}_2$  group. Antibodies to the first antigen will not react with this altered antigen.

Almost any sort of chemical alteration in a protein will alter its specificity. A slight reaction may occur if, instead of substituting a  $-\text{SO}_3\text{H}$  group, we introduce, say, a  $-\text{Cl}$  atom in place of the  $-\text{NH}_2$  group. The antibodies produced in response to the original antigen are said to cross-react with the chlorinated antigen. Many cross-reactions occur between closely related antigens. In a natural example, serum of an animal injected with horse blood will react strongly with horse blood and, to some extent, with mule blood; but not at all with chicken blood. The biological relationship is close between horse and mule, but not between horse and bird.

**Antibodies and Adaptive Enzymes.** While not absolutely alike in every detail, antibody formation and adaptive enzyme formation are remarkably similar phenomena. In each type of response the cells react to a chemical stimulus by forming a complex, protein substance, the molecules of which are so constructed as to combine and interact *specifically*\* with the chemical agent which stimulated their production. When the stimulus is not poisonous, and/or is nutrient in character, we say that an adaptive enzyme has been produced. Antibodies are not all enzymes, but they are very enzyme-like, especially if we regard complement as a sort of kinase or enzyme-activator.

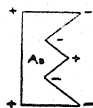
#### ANTIGEN-ANTIBODY REACTIONS

When mixed in a test tube, the antigen and antibody colloids (if they are specific for each other), coming into contact, orient themselves with respect to their positive and negative charges so that an absolute "fit" is obtained. Thus, we may imagine an antigen molecule or particle (Ag) to be represented by the figure:

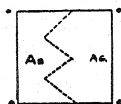


\* There are some minor deviations from strict specificity in adaptive enzyme formation; but there are also some in antigen-antibody relationships.

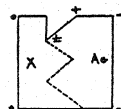
its corresponding antibody (*Ab*) by the figure:



and the antigen-antibody combination by the figure:



The compound colloidal particle, formed as a result of the interaction between these two would be electrically neutral and very large. As a result, it would become unstable in solution and would therefore go out of solution. In a test tube reaction, it would become visible as a cloudy precipitate (Fig. 21-6). This sort of reaction is commonly seen when antibody reacts with a soluble protein or carbohydrate antigen. It is called a "*precipitin reaction*." A weak cross-precipitation might occur between antigen Ag and some antibody (x) closely similar to Ab, but not exactly like it:

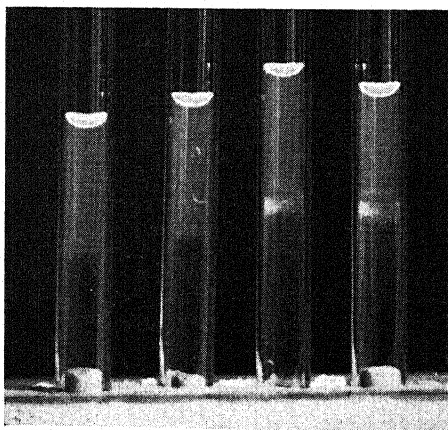


**Stability of Antigen-Antibody Combinations.** A few additional points concerning antigen-antibody reactions are worthy of mention at this point.

1. TYPE OF UNION. *The union of the two is firm but not necessarily irreversible* since they may often be separated by certain procedures. The combination is principally a physical one, depending mainly on adsorption.

2. ANTIGEN-ANTIBODY REACTIONS OCCUR AT SURFACES. Surface structures are in great part the determining factors in antigen-antibody reactions. It is the arrangement of the *surfaces* of molecules, and of cells, and *surface*

Fig. 21-6. Precipitin test in narrow-bore glass tubes. The antigen solution is the cloudy zone above; the antiserum is the clear zone below. At the interface between them, in the two right-hand tubes, a definite white floc or precipitate has formed, representing a reaction between specifically related antigen and antibody. The same antigen fails to react with a different (non-specific) serum in the two left-hand tubes ( $\times 5$ ). (Preparation by Dr. Elaine L. Updyke. Photo courtesy of the Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.)



structure and charges of colloids, which dominate reactivity and specificity. This is of especial importance in relation to cells such as bacteria. Only the surfaces of cells are of significance in reacting with antibodies since antibodies, being colloids, cannot gain access to the interior of the intact cell. Change in chemical or physical structure of the surface of a bacterial cell (for example, presence or absence of capsule) therefore determines whether or not it will react with a particular antibody for that cell.

3. ANTIGENS MAY BE INTRACELLULAR. Although only the surfaces of antigens react with antibodies, the antigens in the interior of complex structures like bacteria stimulate production of antibodies because the bacteria are disintegrated in the body and their intracellular proteins, etc., are released. Thus, a serum may contain a number of antibodies stimulated by a bacterium but only that which is specific for the bacterial *surface* antigen can act.

4. ANTIGEN-ANTIBODY REACTIONS OCCUR IN STAGES. The first is *combination*, presumably due to adsorption (or accumulation) of antibodies on antigen surfaces. This stage may proceed rapidly at temperatures around 37° C. The second stage is *visible reaction* (precipitation, cell lysis or other effect). This stage often develops slowly and may be demonstrated best after twelve to eighteen hours at 4 to 6° C. The presence of electrolytes (MgCl<sub>2</sub>, NaCl, etc.) and of certain other, enzyme-like components of serum (complement) (to be described farther on in this chapter), is often necessary for the second stage.

**Factors Influencing Development of Active Immunity.** MALNUTRITION. Faulty nutrition has long been known to lower general health, strength, and resistance to disease. This is a non-specific factor. Nutrition is thought by some to have some effect also on specific immunity although the evidence for this is generally rather confusing and not conclusive. Some studies indicate that deficiency of certain vitamins interferes with specific antibody formation.

IRRADIATION. Irradiation with x-rays is well known to lower the number and/or effectiveness of phagocytic cells in the blood and in the reticuloendothelial system. It also appears to suppress formation of antibodies. It has already been mentioned that irradiation lowers properdin levels. Infection occurring post-irradiation is therefore likely to progress.

Bacteria of intestinal origin are not held in check in irradiated animals so that fatal bacteriemia by the intestinal hordes is important as a cause of death following excessive irradiation.

CORTISONE OR ACTH. This hormone, derived from cells forming the cortex of the adrenal gland, is sometimes used for treatment of certain diseases of the supporting and connective tissues of the body. These diseases are sometimes manifested in joints (arthritis). This hormone has a number of physiological effects, many of which are little understood, some unknown and some dangerous. One of these effects is diminution of circulating antibodies. Cortisone appears to suppress antibody formation, both during the antigenic stimulus and afterward. This may be related to the well-known power of cortisone to inhibit or suppress inflammatory reactions, but the mechanisms involved are obscure.

**Antigens in Nature Are Usually Mixed.** In Nature, antigens seldom occur as pure compounds. This greatly increases the difficulties of the study of antigen-antibody reactions involving such antigen mixtures as bacterial cells.

A bacterial cell may consist of many antigens (cytoplasm, flagella, capsules, etc.). The serum of a person or animal, following injection with such cells, contains a mixture of antibodies specific for each separate antigen.

To complicate matters further, the cells of two different species, whether closely or distantly related, may contain certain antigens in common so that antiserum\* prepared by injecting cells of one species will react with both kinds of cell.

For example, three related species of dysentery bacilli may each contain four antigens as follows:

Species I	<u>A/B/C/D</u>
Species II	<u>C/D/E/F</u>
Species III	<u>E/F/G/H</u>

Obviously, upon injecting species I into a rabbit, antibodies a, b, c, d, will be engendered, corresponding to antigens A, B, C, D. Upon injecting species II into another rabbit, antibodies c, d, e, f, will be called forth. Likewise, species III will stimulate production of antibodies e, f, g, h. Now, the serum of rabbit I will react best of all with bacterial species I when these bacteria and serum of rabbit I are brought into contact. Serum II will similarly react best with species II, and serum III with species III. However, since serum I contains antibodies c and d, it will cross-react to some extent with cells of species II, since the latter have these antigenic compounds in common with species I. There will be no cross-reaction between serum I and antigen III, but serum II will cross-react with species III. Such cross-reactions are commonplace.

**Antibody Adsorption.** If a given volume of serum I (say 5 ml) be mixed with a heavy suspension of cells of bacterial species II, then antibodies c and d, and antigens C and D will combine, leaving antibodies a and b still free in the serum. By means of centrifuging, the bacteria with their attached antibodies c and d can be removed, leaving serum I free from antibodies c and d and *specific* with regard to species I; i.e., the serum will no longer cross-react with species II and will react only with bacteria containing antigens A and B. If we further adsorb antibody b by treatment of the serum with some species having only antigen B in common with species I, then we obtain a pure A serum.† Many such pure and specific sera are thus prepared and it has been possible to make very extensive analyses of the antigenic structure not only of bacteria, but of higher plants and animals, and to detect antigenic relationships hitherto unsuspected.

Even such unrelated organisms as type XVI pneumococci and the erythrocytes of certain human beings contain antigens common to both. These are detectable by just such methods as those indicated in the preceding paragraph. Students of evolution may not be humiliated to learn that their blood contains antibodies related to proteins in certain fish. The relation of cow to whale by immunological methods is perhaps an unexpected revelation. While this may not be so evident, who can doubt the relation of horse to mule?

\* Serum containing antibodies.

† Such a serum is said to be monovalent.

Methods like these are of immense value in unraveling complex evolutionary lines and species relationships.

**Labeled Antibodies.** ISOTOPES. One of the most interesting technical advances in the field of immunology is the "labeling" of antibodies so that their place and activity can be observed. Antibody proteins may be combined with radioactive isotopes; for example  $I^{131}$ . Material treated with the radioactive, specific antibody solution (infected tissues, suspensions of microorganisms, soluble antigens, etc.) takes up the labeled antibody, if there is a specific antigen present to react with it. By means of a device like a Geiger counter the occurrence of a specific antibody-antigen combination can be detected and the location and amount of specific antibody can be determined with a useful degree of accuracy.

**FLUORESCENT\* ANTIBODIES.** Another exceedingly valuable and important means of labeling antibodies is to conjugate the antibody protein with a fluorescent dye (usually fluorescein). Let us suppose a single bacillus of a certain species is suspected to be present in a large section of tissue from an infected patient. It is impossible to find the one tiny organism, even though gram-stained, because the large mass of surrounding tissue also takes the stain and the bacillus is lost like the needle in the proverbial haystack.

Let us flood the tissue section with *specific*, fluorescein-labeled antibody. Fluorescent antibody attaches itself to the *single*, hidden bacillus. Then we wash out all of the unattached antibody. When the tissue section is illuminated with ultraviolet light, the hidden bacillus reveals itself by its brilliant, yellow, fluorescent light like the full moon at midnight (Figs. 21-7, 21-8). This method will eventually acquire an international importance.

#### ANTIGENIC STRUCTURE OF BACTERIAL CELLS

**Somatic (O) Antigens.** Soma is the Greek word for body. We may think of the cell wall and its contents as the body or *soma* of a microorganism. Somatic antigens, therefore, are those associated with the cell wall or interior of the cell. They are sometimes very poisonous proteins and are, therefore, often called *endotoxins*, meaning toxins inside the cell. They are characteristically *unaffected by boiling*.

There are often several kinds of somatic antigens per cell. Somatic antigens may be quite distinctive of a given species of cell. As often happens, however, the same protein may be found in several, closely related species or even in very remotely related orders or even kingdoms. For this reason, these shared antigens are often spoken of as *group antigens*.

Somatic antigens are often called *O antigens*. (See also Chapter 16.)

**Flagella (H) antigens** are localized in the flagella of motile species. In contrast to somatic antigen they are destroyed by boiling. They often exist in one of two different degrees of specificity called *phases*. In the *specific phase*, phase I, they are specific for the species in which they occur. In the less specific or *group phase*, phase II, they resemble antigens in one or more closely related species or types.

They appear to vary, often unpredictably, from one phase to the other for

\* By fluorescence is meant the property possessed by certain substances of reflecting rays having a wave length different from that of the incident rays.

Fig. 21-7. Use of fluorescent antibodies to locate specific antigen in measles-infected, monkey-kidney cells. Antibodies from a measles convalescent have been conjugated with a fluorescent dye and applied to the tissue cells. Free, excess dye has been washed away. On viewing the tissue cells in the microscope with ultraviolet light the antibodies are clearly seen by their brilliant, yellow-green fluorescence (light areas in the photograph). These fluorescent areas represent specific antigen-antibody combinations. They are seen especially in masses in (or on) the cell nuclei and and, to a lesser extent, in the cytoplasm. ( $\times 700$ .) (Microphotograph courtesy of Drs. S. M. Cohen, I. Gordon, F. Rapp, J. C. Macaulay and S. M. Buckley, in Proc. Soc. Exp. Biol. and Med., 1955, vol. 90.)

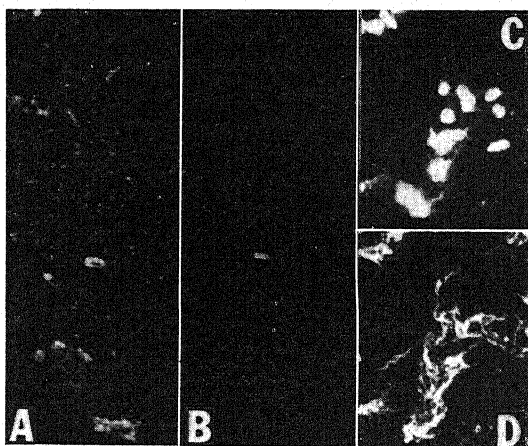


Fig. 21-8. Use of fluorescent antibody to detect specific antigen-antibody combination in mixed materials. *A* shows a sample of soil infected with *Malleomyces pseudomallei*, a dangerous, pathogenic bacterium. A smear of the infected soil, on a slide, has been treated with fluorescent antibody specific for *M. pseudomallei*. Illuminated by ordinary light (dark-field illumination) (*A*) it is impossible (without glancing at *B*) to distinguish the single cell of *M. pseudomallei* from the many saprophytic soil bacteria and soil particles which are present in the sample. In the same specimen, illuminated by ultraviolet light (*B*), combination between the fluorescent *M. pseudomallei* antibodies and the single cell of *M. pseudomallei* present in the mixture is strikingly evident. *C* shows typhoid bacilli, treated with fluorescent antibodies specific only for the flagella of this species. Illuminated by ordinary light the flagella are not very prominent and there is no distinction in visibility between the flagella and the bacilli to which they are attached. In *D*, illuminated by ultraviolet light, the specificity of the combination of fluorescent H antibody with flagella only is clearly demonstrated. The rapidity and specificity of this method of detecting pathogenic microorganisms, or of particular structures in organisms, are obvious advantages over prolonged, laborious and uncertain cultural and ordinary staining procedures. (Courtesy of Drs. B. A. Thomason, M. D. Moody and M. Goldman, in J. Bact., 1956, vol. 72.)

various reasons, some known, some not. This is spoken of as *phase variation*. This is of great importance in identifying certain bacteria, especially certain intestinal pathogens (notably the genus *Salmonella*). There may be several different flagellar antigens in a given organism.

Flagellar antigens are, for reasons previously explained (Chapt. 16), often called *H antigens*.

**Capsular (K) Antigens.** These are usually polysaccharide in composition and are situated in (or wholly compose) the capsule. In those species which possess capsules, the capsular substance dominates the surface and often determines antigenic specificity of a very high order. The localization of antigenic specificity in the capsular substance of such organisms as pneumococci, influenza bacilli, streptococci and Friedländer's bacillus is a striking phenomenon. Stripped of this specific surface antigen, the exposed antigens of the naked cells cross-react very widely with antisera of related and often unrelated species. For example, the somatic or cellular antigens of all pneumococci are immunologically alike. Antibodies for one react equally well with all. But the antibodies of the capsules are not all alike and it has been found that there are about 75 different very distinct types of pneumococcus capsular antigens. Each antigen determines a different type of pneumococcus. Similar series of capsular types are found in influenza bacilli (*Hemophilus influenzae*, types A, B, C, D, E); of meningococci (*Neisseria meningitidis*, types I, II, II alpha) and a number of other species of microorganisms having important agricultural, industrial and geological relationships.)

**Extracellular Antigens.** Some of the waste products produced by living cells are antigenic. For example, diphtheria and tetanus toxins are waste proteins excreted extracellularly. Toxic, extracellular antigens like diphtheria toxin are called *exotoxins*.

#### TYPES OF ANTIBODY RESPONSE AND REACTION

There are different manifestations of antigen-antibody reaction, some of them demonstrable in test tubes as visible reactions, some of them not visible except indirectly by secondary tests. The different manifestations are spoken of as though different antibodies were involved, but there are probably fewer antibodies than types of reaction. The kind of reaction seems to depend in great part on the kind and size of antigen molecule or cell, the physical conditions of the suspending fluids, the presence of electrolytes, and other factors. For convenience, we shall speak of "types of antibody," including in that term "types of antigen-antibody reactions." Among the best understood types of antibody or antigen-antibody manifestations are *antitoxins*, *cytolysins*, *agglutinins* and *precipitins*. Other antibodies (or antibody manifestations) to be considered are so-called *immobilizing antibodies* and the *immune adherence phenomenon*.

**Antitoxins.** When bacteria gain a foothold in the body and secrete toxin into the blood, the toxin, being a protein antigen, stimulates the production of antibody. This antibody neutralizes the toxin and is therefore called *antitoxin*. The reaction may be thought of as a precipitin reaction in which the particles of precipitate, as a rule, are too small to be seen. Under certain conditions in which the quantitative relations are carefully adjusted, visible flakes or floccules are produced. The reaction is quantitative but seems to obey

primarily the laws which govern physical adsorption rather than chemical combination. The toxin-antitoxin reaction is obviously a kind of precipitin reaction.

**QUANTITATIVE RELATIONS (THE FLOCCULATION REACTION).** As mentioned above, when exactly the right *proportions* of toxin and antitoxin are brought together in a test tube a visible precipitate or flocculation occurs. This fact is made use of in determining the concentrations or "strengths" of toxin or antitoxin. For example, we may set up a row of ten tubes. In the first we may put serum containing a quantity of diphtheria antitoxin arbitrarily spoken of as 2 units.\* In the next we place 4 units, in the next 6 units, and so on. We then add to each tube a fixed amount, say 1 ml of filtered broth culture which contains diphtheria toxin in unknown amount. After a short time flocculation appears in one of the tubes, let us say the sixth tube. Since this contained 12 units of antitoxin, we have a measure of the potency of the toxin broth. We say that it contains 12 flocculation units ( $12L_f$ ) of toxin per ml. This is an arbitrary unit of potency and is used for convenience to define diphtheria, tetanus and other toxins. It shows combining and flocculating potency but not necessarily toxicity. Partly deteriorated toxins (toxoids) will still give the same, undiminished  $L_f$  value.

Conversely, by using a series of tubes containing known, graded amounts of toxin, we may determine the number of  $L_f$  units of antitoxin in a serum of unknown potency.

**ZONE REACTION.** The above reaction illustrates a curious but common manifestation called a *zone phenomenon* (sometimes called pre-zone and post-zone, or zones of inhibition).

Observe in the above experiment that flocculation failed to occur in a zone of 5 tubes before the 6th tube and in a "zone" of 4 tubes after the 6th (pre-zone and post-zone). This phenomenon depends in part on the fact that for maximal amounts of visible precipitate (or flocculation or other antigen-antibody reaction) to occur most rapidly optimal *relative proportions* of antigen and antibody must be present. If there is too great an excess of either, less precipitate, or none at all, will appear. When we use a series of graded quantities of antibody serum with a fixed dose of antigen, we might expect the highest concentrations of antibody to give the strongest reactions, the smaller quantities correspondingly less. This is a common experience. However, often the zone phenomenon is manifest and no reaction occurs with the greater, or with the weaker, concentrations but only with the middle concentrations. Many factors are involved, among which are type of animal serum used, nature of antigen and antibody, pH, electrolytes, and so on.

**"BLOCKING ANTIBODIES."** Pre-zones may also occur due to the presence of partial, imperfect or partly deteriorated antibodies. These are sometimes called "toxoids," "agglutinoids" or "blocking antibodies." They have the ability to combine with the antigen, to the exclusion of perfect, complete antibodies. They are, themselves, incompetent to cause whatever distinctive manifestation is involved. When they are present they prevent the true antibodies from acting.

\* A unit (*approximately*) of diphtheria antitoxin is the least amount necessary to protect standardized (250 to 300 gm) guinea pigs against 100 minimal lethal doses (M.L.D.) of diphtheria toxin. An M.L.D. kills about 75 per cent of such pigs in from four to five days.



They do not manifest themselves in the higher dilutions of antibody-serum because the defective antibodies are made too dilute to interfere; i.e., they are "diluted out."

**Haptenes.** This term, or partial antigens, is applied to substances which are related to antigens, but are not actually capable of stimulating production of antibodies. They can, however, *combine* with antibodies, thus blocking or *excluding* the complete antigen. A visible manifestation of the combination may occur but generally does not. The situation is the reverse of that seen in blocking antibodies.

Thus, zone phenomena may be produced by partial or imperfect antibodies or partial or imperfect antigens (haptenes).

The practical importance of zone phenomena is that, in determining the activity of any antibody-containing serum or antigen by testing it in a series of dilutions, the series should be made sufficiently long, and the intervals between dilution steps not too great, or the zone of optimal proportions may either not be reached or passed over. Dilutions must be sufficient to eliminate partial, or blocking, antibodies as well as haptenes. The same considerations hold for most other serological tests involving antigen-antibody reaction.

**Cytolysins and Complement.** The somatic antigens of any given bacterial cell may call forth antibodies which assist in the lysis of that cell. These antibodies are termed *cytolysins*. They are sometimes called *sensitizers* (see below).

The action of cytolysin may be described briefly as follows: the cytolytic antibody first combines with the foreign cell that called it into being, probably by adsorption on the surface. The cell may be a bacterium, an erythrocyte or a cell of any other nature.\* This simple combination is, however not sufficient to destroy the cell. There is no visible reaction.

**Complement.** A second substance, called *complement*, which is normally present in all mammalian blood and which is entirely non-specific, is necessary to complete (hence *complement*) the lytic action. It, too, combines with the cell which, in order for it to act, must already have been *sensitized* by the cytolytic antibody. Lysis then results.

Complement behaves as though it might be a partial enzyme. As in the properdin system certain ions must be present:  $Mg^{++}$  and possibly  $Ca^{++}$ . Complement, like properdin, is inactivated by zymosan. It is also inactivated by a few minutes' exposure to  $60^{\circ} C$  and a few hours at  $37^{\circ} C$ , by shaking, by certain chemicals and by other factors which inactivate enzymes.

Complement cannot, by itself, destroy foreign cells, but must act through the intermediation of the sensitizer. The sensitizer is a specific antibody, but complement is non-specific; it helps *any* sensitizer to complete its work.

**COMPLEMENT FIXATION.** After complement has combined with the sensitized cell the complement is no longer active. It is said to be fixed. It is adsorbed on the sensitized cells. Complement fixation is the basis of several valuable tests used in the study of microorganisms, disease, immunology, etc.

**THE COMPLEMENT-FIXATION REACTION.** The fixation of complement was first demonstrated in 1901 by Bordet, a famous Belgian scientist. Bordet

\* Not all types of cells appear to be equally subject to cytolysis in this manner.

discovered that if the serum of a person who has recovered from bubonic plague (and therefore containing cytolytins specific for *Pasteurella pestis*, the plague bacillus), were mixed with plague bacilli, the free complement in the serum was all used up (fixed) in destroying the bacilli. When the complement was tested for by an appropriate method, none was to be found free in the serum.

Complement fixation may occur in various other antigen-antibody reactions. Many of these do not necessarily result in cell lysis. This is because complement is readily adsorbed on the surfaces of any finely divided particles, visible or invisible, whether they be immunological precipitates, agglutinations, proteins or inert colloidal particles such as clay or powdered animal charcoal. For example, complement is adsorbed (fixed) by the floccules resulting from the interaction of toxin and antitoxin described above. Complement may thus be fixed in any antigen-antibody reaction but is not a *necessary* component of such reactions as it is in cytolysis.

The phenomenon of complement-fixation enables an investigator to identify the antigens (microorganisms) causing certain diseases, or to detect and identify the corresponding antibodies which appear in the blood as a result of disease. For example, if antibody, antigen and complement are mixed in a tube, we can determine whether antigen and antibody have combined by testing to see whether the complement has been fixed or not. If complement has been fixed, then we know that an immunologically specific, antigen-antibody reaction has occurred. Knowing the identity of antigen or antibody, we can identify the other.

*The Wassermann Test.* A well-known use of the complement-fixation test in this manner is in the diagnosis of syphilis. It was first described by Wassermann. It is subject to serious errors due to non-specificity of "artificial" antigens used in it. (See Chapt. 31.)

**Immobilizing Antibodies.** The etiological (causative) agent of syphilis is a spirochete, *Treponema pallidum*. The organism is actively motile, rotating on its long axis and bending and flexing vigorously. It may also have flagella. In the serum of patients with syphilis *T. pallidum-specific* antibodies appear some days or weeks after initial infection. These antibodies promptly *immobilize* and *kill* the spirochetes within a few hours when mixed with them in test tubes. This effect is readily seen by examining the mixture with a dark-field microscope. It is commonly spoken of as the TPI (*T. pallidum immobilization*) test. The immobilizing action does not take place unless complement is present. Curiously, little or no complement is fixed in this reaction. The role of the antibody is evidently that of a sensitizer, but the action of the complement is not so obvious, as no lysis or other visible effect (except death and loss of motility) occurs.

The TPI test is one of the most important immunological developments in syphilology. It is the first *specific* serological test for the disease. When technical difficulties (which at present interfere with its *general, routine* use) are overcome, it will help to replace non-specific serological methods commonly used for the diagnosis of syphilis. These difficulties are being rapidly eliminated.

Immobilization by specific antibodies also occurs in other microorganisms. This is readily seen in *Entamoeba histolytica* (the cause of amebic dysentery)

when the active trophozoites of this protozoan are treated with the serum of dysentery-immune animals. Similarly, there are specific immobilizing antibodies for the ciliated larval state (*miracidium*) of a pathogenic worm (the fluke, *Schistosoma mansoni*) and for motile bacteria other than *T. pallidum*.

In the immobilization phenomena other than the TPI, complement is not always necessary and death of the organisms does not necessarily follow immobilization. They may, on the contrary, recover completely.

**The Immune-Adherence Phenomenon.** This, also, is an immunologically-specific reaction in which various species of bacteria, sensitized with specific antibody, *adhere strongly to erythrocytes*. Complement is essential to the immune-adherence.

A very important feature of this reaction is the fact that the microorganisms adhering to erythrocytes are much more readily and actively phagocytized. This is another illustration of the important relationship between phagocyte and antibody.

**THE PARTICULATE ADHESION PHENOMENON.** The immune adherence phenomenon just described is probably a particular manifestation of the more general *particulate adhesion phenomenon*. Certain antibodies affect the surfaces of organisms for which the antibodies are specific by making them sticky. As a result, not only homologous bacteria stick together (specific agglutination) but non-specific particulate matter of various sorts sticks to them: leukocytes, erythrocytes, heterologous bacteria, gamboge, collodion, yeast cells, etc. Complement appears to be involved in the particulate adhesion phenomenon whereas it is not essential to specific agglutination.

**Agglutinins.** In addition to antitoxins, cytolytins, immobilizing antibodies, immune adherence, and complement, there are antibodies which cause bacteria to stick together in flocks or clumps as though they were coated with some glutinous substance. Such antibodies are called *agglutinins*. Like all other antibodies, they are specific. They do not necessarily kill bacteria but aid the leukocytes by gathering the latter's prey into groups. A leukocyte, or other phagocytic cell, can engulf 50 agglutinated bacteria fifty times as easily as 50 separate ones, and in much less time. Furthermore, the agglutinins appear to *opsonize* the surfaces of the bacteria so that the phagocytes can grasp and engulf them more readily.

**DIAGNOSTIC USE OF AGGLUTININS.** Agglutinins are very widely used in the identification of bacteria and the diagnosis of disease. Let us assume that a patient has a febrile disease which has remained undiagnosed for a week or more, during which time antibodies have formed. We draw a little blood from a vein and allow it to clot. We then remove the clear serum and mix it, suitably diluted (1:20, 1:40; 1:80; . . . 1:2560), in a series of test tubes with, for example, a suspension of typhoid bacilli (*Salmonella typhi*). These bacteria are commonly maintained in diagnostic laboratories for this purpose. If the patient has typhoid fever his serum will contain typhoid agglutinins and the bacilli will be found in flocks or clumps (Fig. 21-9). If no agglutination occurs, either the patient does not have typhoid fever or has not yet had time to develop antibodies. However, antibodies can usually be detected after a week of fever. Another test will be made later.

This means of diagnosing typhoid fever is called the *Widal reaction* after

Widal, who first published upon the subject. The term *Widal test* is sometimes (improperly) applied to *any* agglutination test.

**IDENTIFICATION OF BACTERIA BY THE AGGLUTINATION REACTION.** Conversely to the Widal test, in the identification of an unknown organism, sera containing various *known* antibodies are mixed with suspensions of the unknown bacterium, and agglutination is looked for. Suppose, for example, that we have a gram-negative rod which, by its cultural reactions, we know to belong to the typhoid-dysentery group. We may, as a preliminary test, set up two series of tubes: *A*, containing serial dilutions of serum of a typhoid-immune animal; *B*, containing dilutions of serum of a dysentery-immune animal. A drop of our "unknown" bacterial suspension is added to each tube. If, after several hours, no change has occurred in the first series of tubes while the serum in the tubes of series *B* has caused the bacilli to fall to the bottom of the tubes in clumps, we know that, since the serum in *B* contained only dysentery agglutinins, our unknown organism must be some species of *Shigella* (dysentery bacilli). Many other bacteria, saprophytic and parasitic, of agricultural, industrial and other special interests, may be identified in this way.

**THE HEMAGGLUTINATION REACTION.** This procedure, widely used in immunology, illustrates very nicely the fact that antigens at the *surface* of a cell determine its immunological specificity. Erythrocytes (sheep, cow, etc.) are washed free from their serum and suspended in a saline solution containing any desired *soluble* antigen; for example, an antigen extracted from tuberculosis bacilli. This antigen is adsorbed by the surfaces of the erythrocytes and covers them as a coating. The cells are removed from this suspension and excess antigen is removed.

A series of dilutions is now made with serum containing antibodies specific for the tuberculosis antigens with which the erythrocytes are coated. Into each serum dilution is introduced a drop of the suspension of antigen-coated erythrocytes. These behave as though they were tubercle bacilli! Within a short time the coated sheep erythrocytes are agglutinated. Tuberculosis antibodies have no visible effect whatever on the *normal*, untreated erythrocytes of a sheep. It is interesting that *totally inert* particles of plastic, gum arabic, etc., can be similarly coated with antigen and agglutinated by antibodies.

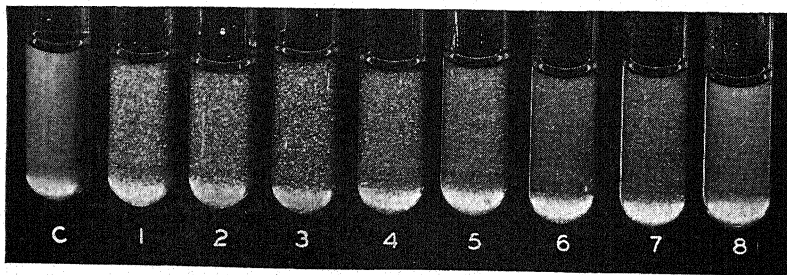


Fig. 21-9. Macroscopic agglutination test. Control tube *C* contains bacterial suspension only. Numbered tubes contain bacterial suspension plus the following dilutions of serum: 1:100, 1:200, 1:500, 1:1,000, 1:2,000, 1:5,000, 1:10,000, and 1:20,000. Agglutination is evident in dilutions 1:100 to 1:10,000 (tubes 1 through 7) but not in 1:20,000 (tube 8). The titer of the serum is therefore 1:10,000. (From Burrows, Textbook of Microbiology.)

**VIRAL HEMAGGLUTINATION.** Many viruses have the property of causing hemagglutination. This appears to be due to the existence of multiple, specific receptors on erythrocytes and/or viruses. One virus particle can thus combine with more than one erythrocyte simultaneously and vice versa, thus causing visible clumping of the erythrocytes. This is often referred to as Hirst's phenomenon after its discoverer.

The phenomenon is commonly used in diagnosis to titrate the virus content of virus-infected fluids such as serum, nasal washings, and fluids from experimentally infected chick embryos. The hemagglutinating powers of serial dilutions of such fluids are tested. The amount of hemagglutination produced by each dilution is recorded and the result may be expressed in terms of "units" of virus present in the fluid.

**HEMAGGLUTINATION INHIBITION (HI).** If the specific receptors of the virus particles are preempted by specific antibodies, obviously they cannot combine with erythrocytes. Viral hemagglutination is thus specifically inhibited by antibodies.

It is commonplace diagnostic procedure to measure the HI power of antibody-containing fluids (for example, serum of patients). Serial dilutions of the patient's serum are mixed with standardized doses of the virus being studied. After allowing time for combination, equal amounts of a standardized erythrocyte suspension are added to each tube. The extent to which hemagglutination is inhibited gives a measure of the concentration of specific antibodies in the serum.

**Precipitins.** We have previously described the precipitin reaction, using it as a model of a typical antigen-antibody reaction. Precipitins seem to be very closely related to agglutinins and are probably merely a different manifestation of the same antibodies. They cause the clumping and precipitation of invisible molecules of protein (instead of whole cells) so that a visible turbidity of flakiness is formed.

**USES OF PRECIPITINS.** Because of the very high degree of specificity of precipitin reactions, serological differentiation between soluble proteins of closely similar composition is possible, a feat impossible of attainment by chemical means.

An interesting application is seen in the use of precipitin tests to determine the animal (host) from which a mosquito had its most recent blood meal.

In determining mosquito-host blood, one first catches his mosquito (full of the blood to be tested). The mosquito is crushed in 1 or 2 ml of saline solution and the blood is thus extracted. This constitutes the antigen to be tested. The sera with which to test it are previously prepared in rabbits by injecting the rabbits with blood from various animal species. One rabbit receives bovine blood, another equine blood, and so on. The serum of each rabbit contains precipitins against a certain species of animal. By bringing into contact a little of the mosquito extract (antigen) with each of the rabbit sera (specific antibody) in turn, one serum will be found which causes a definite precipitation. If that serum is from a rabbit immunized with bovine serum, then we may say the mosquito probably got its blood meal from one of the nearby cattle. This information is of use in the control of mosquito-borne diseases. It guides efforts toward eradication of the mosquitoes which bite *man*.

**THE PRECIPITIN TEST APPLIED TO SYPHILIS.** It seems to be well established

that, in complement fixation tests, the complement is fixed because it is adsorbed onto finely divided particles of antigen-antibody precipitate. In the Wassermann test for syphilis, the precipitate formed is not visible. It would be much better if we could avoid having to test for the presence of this invisible precipitate by adding complement and then being forced, in turn, to test for the presence of complement by adding sensitized red corpuscles. It would be a great advantage if we could see the precipitate directly as in other precipitin reactions. This has been accomplished.

A specially prepared, and very concentrated, alcoholic antigen is used in which the reactive substances are present, but in the form of large, unstable, colloidal complexes. These are brought (by proper dilution with saline solution), to a state where, in contact with syphilitic serum, they precipitate in a visible form. Generally, no precipitation occurs in the presence of normal serum *under proper circumstances*. Tests based on this principle are the *Kahn* test, *Eagle* test, *Hinton* test, *Mazzini*, *V.D.R.L.*, etc.

✓ THE UNIVERSAL SEROLOGIC REACTION. The antibodies concerned in these tests are not related to syphilis at all but are produced by deteriorated tissues resulting from normal "wear and tear" of the body. Certain materials inside of such normal but "worn-out" tissue cells appear to act as antigens which call forth antibodies (precipitins). Similar antigens may be extracted from the tissues of normal animals. The tissue antigens are found especially in heart muscle and are lipid in nature. Commercial antigens commonly used in testing for syphilis are, therefore, derived from beef hearts and are called cardiolipins. They can give false positive reactions, with always distressing, sometimes tragic, results. The advantages of tests using *specific* antibodies, previously mentioned, are therefore obvious.

Now, there are 3 controlling factors in the precipitin test for syphilis using these antigens: (a) temperature, (b) concentration of NaCl in the saline diluting fluid and (c) dilution of the serum to be tested. By suitable arrangements of these factors the serum of syphilitic persons usually can be made to give a positive precipitin test with the cardiolipin (and similar) antigens. By making other adjustments, the reaction can be made to occur in normal serum. By making still other adjustments, the reaction is found to occur in tuberculosis, malaria and leprosy and also in different species of normal animals. Because of its wide occurrence under proper adjustments of the test procedure, the phenomenon has been called the *Universal serologic reaction*.

**Antigen-Antibody (Precipitin) Reactions in Gels.** A very important method of demonstrating precipitin reactions makes use of *agar gels*. Reactions not demonstrable by other methods can readily be made visible by this method. To show the reaction, one mixes a specific antiserum with warm, fluid agar, half fills a narrow tube with it and allows it to solidify. The corresponding antigen, which must be in solution (not cellular), is placed in contact with the agar. As the antigen diffuses downward into the agar, a white band of precipitate appears in the agar at the zone of optimal proportions. If the antigen is a pure substance, only one band appears. If the antigen is a mixture, and the serum contains antibodies for each antigen in the mixture, then several bands may appear, corresponding in number to the number of antigen-antibody systems present. The possible value of this technique in detecting impurities in antigens, in protein analysis, etc., are evident. Here is a research

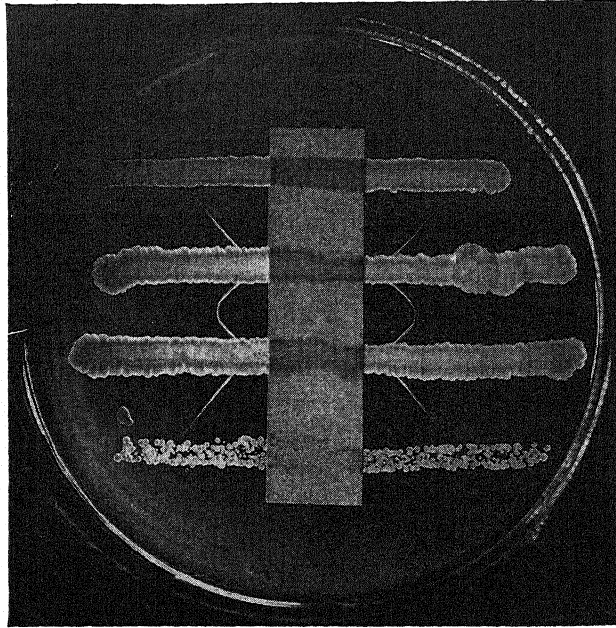


Fig. 21-10. The in vitro test for virulence. Serum agar was poured into the dish at about  $45^{\circ}\text{C}$ . Before it hardened, the strip of filter paper, saturated with diphtheria antitoxin, was pressed to the bottom of the agar in the dish. After the agar hardened it was inoculated on the surface in long streaks at right angles to the paper. As the growth developed, at  $37^{\circ}\text{C}$ , toxin diffused from the culture into the agar. Simultaneously, antitoxin diffused from the paper strip. Where the toxin and antitoxin met, in proper concentration for reaction, precipitation occurred. This is seen as thin white lines between the growths of the cultures. This reaction with diphtheria antitoxin is produced only by toxigenic *C. diphtheriae*. The growths nearest the ends of the strip of paper are not *C. diphtheriae*. (Specimens prepared by Miss Elizabeth O. King. Photo courtesy of the U. S. Public Health Service, Communicable Disease Center, Atlanta, Ga.)

tool, simple and inexpensive to use, which should prove attractive to any scientifically curious student.

**THE IN VITRO TEST FOR TOXIGENICITY.** A very useful application of the principle has been made to studies of the toxigenicity of diphtheria bacilli *without the use of animals*. A strip of sterile filter paper saturated with diphtheria antitoxin is embedded in the center of a plate of nutrient agar, while the agar is still warm and fluid. After the agar has solidified, the culture of diphtheria bacilli to be tested is inoculated in a single straight streak across the surface of the agar at an angle of  $90^{\circ}$  to the strip of paper. The plate is then incubated.

The diphtheria antitoxin diffuses through the agar from the paper strip in diminishing concentrations, an infinite series of dilutions, from the paper outward. Diphtheria toxin, given off by the growth on the agar surface, likewise diffuses into the agar, but at right angles to the direction of diffusion of the antitoxin; also, in an infinite series of dilutions. Wherever the two meet *in optimal proportions* for precipitation to occur, a white point appears in the agar. Theoretically, all of these points should occur on a line bisecting the

90° angle, more intense near the intersection of paper and line of growth, growing fainter with distance from the angle of intersection. This actually occurs, and all of the points of precipitation coalesce into a single straight line. This is a very pretty "living graph," the paper strip being the origin of the ordinates (concentrations of antitoxin); the line of growth, the origin of the abscissae (concentrations of toxin) (Fig. 21-10).

The method determines toxigenicity (virulence) of diphtheria bacilli, a property hitherto demonstrable only by expensive and time-consuming inoculation of animals. In many instances the *in vitro* test is more dependable than the *in vivo* test. This agar-plate diffusion method, in several ingenious modifications, has been used in many very clever and fruitful investigations of proteins, natural antigens, antibodies, antigen-antibody physical and chemical relationships, etc.

**Protective Antibodies.** All of the immune reactions so far mentioned are demonstrable by *in vitro* methods. It was mentioned that immunity does not necessarily result from the presence of such antibodies. Indeed, it seems that (as previously indicated) most of them aid in the process of phagocytosis. Some are clearly lytic, some antitoxic, some immobilize. The action of others is not demonstrable *in vitro*. The only reliable method of detecting and measuring such antibodies is to infect experimental animals (e.g., mice) and give them doses of the serum to be tested to see whether they are thereby protected. This measures *protective power* directly, regardless of whether this power depends on agglutinins, cytolytins, or some still undiscovered antibody. Such a test is known as a *protection test* and is widely used to measure the antigenic virtues of antigens and the protective power of sera.

#### REFERENCES

- Anonymous: Nonspecific defense mechanisms of the body (the properdin system). *J.A.M.A.*, 1956, 162:1271.
- Boone, I. V., Woodward, K. T., and Harris, P. S.: Relation between bacteremia and death in mice following x-ray and thermal column exposures. *J. Bact.*, 1956, 71:189.
- Boyd, W. C.: *Fundamentals of Immunology*. 3rd ed. Interscience Publishers, Inc., New York, 1956.
- Burnet, Sir M.: How antibodies are made. *Sci. Am.*, 1954, 191:74.
- Carpenter, P. L.: *Immunology and Serology*. W. B. Saunders Co., Philadelphia, 1956.
- Coons, A. H.: Labeled antigens and antibodies. *Ann. Rev. Microbiol.*, 1954, 8:333.
- Cox, C. D.: Hemolysis of sheep erythrocytes sensitized with leptospiral extracts. *Proc. Soc. Exp. Biol. and Med.*, 1955, 90:610.
- Cushing, J. E.: *The Biological Aspects of Immunology*. McGraw-Hill Book Co., Inc., New York, 1956.
- Editorial: Microbes and Metchnikoff. *Am. J. Med.*, 1952, 12:261.
- Elberg, S. S.: Factors affecting resistance to infection. *Ann. Rev. Microbiol.*, 1956, 10:1.
- Gordon, A. S., and others: Leukocytic functions. *Ann. N. Y. Acad. Sci.*, 1954, 59 (Art. 5): 665.
- Gordon, L. E., Cooper, D. B., and Miller, C. P.: Clearance of bacteria from the blood of irradiated rabbits. *Proc. Soc. Exp. Biol. and Med.*, 1955, 89:577.
- Hatch, M. H., et al.: Response of x-irradiated mice to intravenous inoculation of intestinal bacteria. *Proc. Soc. Exp. Biol. and Med.*, 1952, 80:632.
- Haurowitz, F.: The immunological response. *Ann. Rev. Microbiol.*, 1953, 7:389.
- Hollander, A. G., Frobisher, M., Jr., and Kalisch, K.: Clinical evaluation of the hemagglutination reaction. *Am. Rev. Tub.*, 1953, 67:497.
- Lamanna, C., and Hollander, D. H.: Demonstration of particulate adhesion of the Rieckenberg type with the spirochete of syphilis. *Science*, 1956, 123:989.



- Ledbetter, R. K.: The *Treponema pallidum* immobilization test. A diagnostic aid to the clinician. J.A.M.A., 1956, 160:1392.
- Lennette, E. H., et al.: Symposium on newer knowledge of viral and rickettsial diseases. Am. J. Trop. Med. and Hyg., 1956, 5:419.
- Liu, C.: Rapid diagnosis of human influenza infection from nasal smears by means of fluorescein-labeled antibody. Proc. Soc. Exp. Biol. & Med., 1956, 92:883.
- Magnuson, H. J., and Portnoy, J.: The *Treponema pallidum* complement-fixation test. Am. J. Pub. Health, 1956, 46:190.
- Marrack, J.: The structure of antigen-antibody aggregates and complement fixation. Ann. Rev. Microbiol., 1955, 9:369.
- McDermott, W., and others: Natural resistance to infection. Ann. N. Y. Acad. Sci., 1956, 66(Art. 2):233 et seq.
- Miller, J. N., and Carpenter, C. M.: *Treponema pallidum* immune adherence (TPIA) test in diagnosis of syphilis. J.A.M.A., 1957, 163:112.
- Neter, E.: Bacterial hemagglutination and hemolysis. Bact. Rev., 1956, 20:166.
- Nungester, W. J.: Nonspecific factors in immunity. Ann. Rev. Microbiol., 1954, 8:363.
- Raffel, S.: Immunity. Appleton-Century-Crofts, New York, 1953.
- Sawyer, W. D., Smith, M. R., and Wood, W. B., Jr.: The mechanisms by which macrophages phagocyte encapsulated bacteria in the absence of antibody. J. Exp. Med., 1954, 100:417.
- Schwartzman, G., Editor: Symposium No. 6, New York Acad. Med., 1. The Effect of ACTH and Cortisone upon Infection and Resistance. Columbia University Press, New York, 1955.
- Smith, E. L., and Jager, B. V.: The characterization of antibodies. Ann. Rev. Microbiol., 1952, 6:207.
- Suter, E.: Interaction between phagocytes and pathogenic microorganisms. Bact. Rev., 1956, 20:94.
- Talmage, D. W.: Effect of ionizing radiation on resistance and infection. Ann. Rev. Microbiol., 1955, 9:335.
- Tomesik, J.: Antibodies as indicators for bacterial surface structures. Ann. Rev. Microbiol., 1956, 10:213.
- Topley, W. W. C., Wilson, G. S., and Miles, A. A.: Principles of Bacteriology and Immunology. 4th ed. Williams & Wilkins Co., Baltimore, Md., 1955.
- Vennes, J. W., and Gerhardt, P.: Immunologic comparison of isolated surface membranes of *Bacillus megatherium*. Science, 1956, 124:535.
- Wilson, M. W., and Pringle, B. H.: Experimental studies of the agar-plate precipitin test of Ouchterlony. J. Immunol., 1954, 73:232.
- Wright, W. A. and others: Nutrition in infections. Ann. N. Y. Acad. Sci., 1955-1956, 63(Art. 2):145.

# Immunology and Microbiology

## 2. ARTIFICIAL IMMUNITY

IN THE PREVIOUS chapter it was pointed out that persons often become immune to certain infectious diseases by surviving natural attacks of those maladies. The body actively acquires specific resistance against such infections. This sort of immunity is not present in the normal person. Since it is acquired in the course of natural events it is often spoken of as *natural active immunity*. In this chapter we shall speak of purposefully induced immunity, or *artificial active immunity*.

Even though subclinical (or *inapparent*\*) infections often occur naturally, with convenient, painless immunity as a result, natural infections causing immunity are all too frequently very severe, may be disabling and disfiguring, or even fatal. It would be much better if we could become immune by some means which we can control. Thus we could avoid the dangers and discomforts inherent in the natural process. Furthermore, we should like to become safely immune to disease early in life and not have to wait for accidental natural infection, occurring perhaps at a very inconvenient time in adult life. In addition, it is often desirable to be able to produce immunity to certain diseases at certain definite times. For example, a person desiring to do laboratory research with yellow fever virus would like to be able to immunize himself before starting the work, since infection with the virus might otherwise result fatally. So also, physicians and nurses or others working with polio or diphtheria patients should be immunized safely and comfortably against these diseases in time to begin their work. All this, however, is too much to expect of Nature.

In view of these needs, man has devised means of developing specific immunities "artificially" and safely. The methods involve natural processes, but are used under *modified* and *carefully controlled* conditions and are therefore called "artificial immunization." Two types of artificial immunity are used: *active artificial immunity* and *passive artificial immunity*.

\* Inapparent infections are those in which symptoms are so very mild that no special attention is paid to them. The vast majority of infections, fortunately, are of this type.

## ACTIVE ARTIFICIAL IMMUNITY

In active artificial immunity the patient's body is stimulated to develop resistance by being injected with certain kinds of antigens. These are of three general types, as follows: (A) sterile bacterial *exotoxins*; (B) sterile microbial cellular\* antigens (proteins and carbohydrates) in the form of, or derived from, dead microorganisms; (C) living, infectious microorganisms, the virulence of which has been reduced or attenuated by various procedures so that no *serious* infection results.

## PRIMARY AND SECONDARY STIMULUS

Before describing details of artificial active immunization the matter of primary and secondary antigenic stimuli must be gotten in mind. In the ordinary course of life one is constantly being exposed to repeated doses of infectious organisms such as pneumococci, streptococci, and diphtheria bacilli from carriers and from ambulatory, subclinical, mild and inapparent infections. These *repeated antigenic stimuli* serve to keep one's immunity in a good state.

The phenomenon is used in artificial processes also. For example, suppose that a child be given a single dose (1 ml) of diphtheria antigen such as alum toxoid or a "shot" of Salk polio vaccine. In about two weeks his blood, tested by appropriate methods, shows very few antibodies. After four to six weeks, however, his blood is found to contain a satisfactory amount of diphtheria antitoxin, or polio antibodies. The development of immunity, however, has been relatively slow! A year later the amounts of antibodies in his blood are found to have declined to a very low level or to have disappeared entirely. This diminution of antibody concentration in the serum is very common. However, *immunity* has not necessarily disappeared.

**"Booster Doses."** After time has thus reduced the effect of the first antigenic stimulus, let us give the child a second dose of diphtheria toxoid (or polio vaccine) and test his blood for antitoxin (or polio antibodies) at short intervals. A surprisingly rapid and extensive response is now noted. After the first or *primary stimulus* given a year before, response was slow. Response to this dose given as a *secondary stimulus*, occurs at once and in a few hours the child may be found to have one or more units of diphtheria antitoxin (or ample polio antibodies) per ml of blood (Fig. 22-1). In practice, the secondary stimulus is often referred to as a "booster dose."

The body cells react as though, having once had an "antigenic experience," they are more alert and expert to form antibodies of this particular sort and do so with great facility whenever called upon.

Ability to withstand disease largely depends upon this very rapid reactivity. This explains, in part, why resistance to disease, on the one hand, and concentration of demonstrable antibodies in the serum, on the other hand, are not necessarily related.

The principle of the primary and secondary stimulus is a generally applicable one and should be borne in mind. It is not restricted to exotoxins but works equally well with living or dead bacteria, egg white, viruses and virtually all other antigenic substances.

\* Somatic or O, capsular, flagellar or H, etc.

(A) **Immunization by Means of Exotoxins.** Immunization with exotoxins will be readily understood by those who have read the preceding chapter. Cultures of toxin-producing bacteria, like *C. diphtheriae*, are made in broth and, after sufficient growth of the bacteria, are passed through porcelain filters which remove the organisms. The *filtrate* (broth passing through the filter) contains the exotoxin. This may be injected hypodermically (under the skin) in from 1 to 5 very minute doses at weekly intervals into the persons to be immunized. Eventually (usually after two to six weeks) their blood will be found to contain antitoxin which protects them from the toxin in question. This procedure or a modification of it was formerly used in immunizing children against diphtheria. There is considerable danger, however, and many fatal accidents occurred due to overdoses of toxin even when antitoxin was mixed with it.

**Toxoids.** In 1890, von Behring, Frankel and Kitasato discovered that diphtheria and tetanus (lockjaw) toxins, which had been heated for 1 hour at 70° C, were no longer poisonous but could *stimulate antibody production*. The possibilities for the prevention of tetanus and diphtheria were immediately recognized. In 1924, Ramon found that formaldehyde affects the toxins of diphtheria and tetanus much as does heat. Such detoxified but antigenic exotoxin is today spoken of as *toxoid*. In order to avoid the dangers attendant upon the use of toxin, formaldehyde-treated toxoid came into general use for active immunization against diphtheria. The same considerations apply to other exotoxins, such as that of the tetanus (lockjaw) bacillus, etc.

In 1933 Havens and Wheeler found it possible to improve such antigens still further. The addition of alum to broth containing the toxoid *precipitates* the latter and it may then be collected, concentrated and purified to some extent. Alum-precipitated toxoid is highly effective and only two initial injections are needed as compared with three injections of fluid toxoid. Similar discoveries have been made concerning other toxins used in active artificial immunization, notably the toxins of scarlet fever and tetanus. The principle

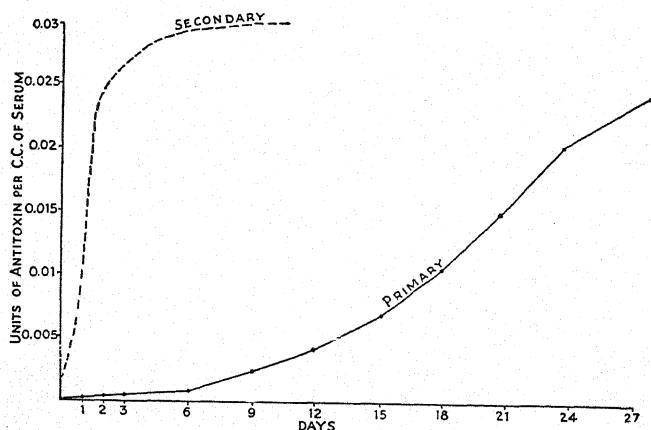


Fig. 22-1. Curves showing rate of antitoxin production following a primary injection and following a secondary injection. Note that after the secondary injection antibody production is much more rapid and extensive than after the first or primary stimulus. The rate and extent of the secondary reaction vary with different individuals, antigens and species.

has also been adapted to bacterial vaccine against whooping cough. It is now common practice to mix several toxoids, as diphtheria and tetanus toxoids as well as bacterial antigens, especially whooping-cough bacilli. The combination of three antigens is even more effective, with respect to each, than any one of the three alone. In addition, the number of separate injections is reduced.

**ACTION OF ADSORBED TOXOIDS.** It is important to understand why alum-precipitated toxoid is more effective than plain, fluid toxoid. When the latter is injected under the skin, the soluble material and fluid are quickly taken into the body fluids and destroyed and eliminated inside a few hours. The antigenic stimulus is very transitory, and repeated injections are necessary. If the toxoid could be held *in situ* for several days, being released continuously, little by little, into the body, the antigenic stimulus would be prolonged and continuous and there would be less need for further injections.

This is accomplished by means of the alum-toxoid. The toxoid is *adsorbed* and held on the surfaces of the alum precipitate, which is insoluble. The alum precipitate remains where injected, undissolved, releasing its adsorbed toxoid slowly, little by little, giving the patient a prolonged and continuous antigenic action which is highly effective.

This principle is not confined to diphtheria immunization but is of broad significance. For example, in a modified form it is used with antibiotics to maintain a high concentration of antibiotics in the blood over a long period without repeated injections. The persistence in the body of any antigen, living or dead, maintains immunity by its continuous antigenic action.

**(B) Immunization with Dead Microorganisms.** A second procedure of artificial active immunization is very similar to the foregoing except that, instead of using excreted growth products (e.g., exotoxin) of bacteria, the microorganisms themselves are used. For convenience, greater yield, and avoidance of foreign matter the microorganisms are usually cultivated on agar (if bacteria), or in suitable fluid medium. In the case of viruses (e.g., polio virus) the microorganisms are cultivated in tissue cultures. For rickettsiae, live chick-embryo yolk sacs are much used.

In dealing with bacteria, the growth on agar is removed to physiological saline solution. Whether bacteria, virus or rickettsiae, the fluid containing them is then heated to about 60° C (or irradiated with ultraviolet light, or treated with formaldehyde or other substances) to kill the microorganisms.

A very minute amount (0.25%) of phenol, tricresol, or some other antimicrobial agent is added to insure sterility.

Such suspensions of killed microorganisms are frequently referred to as vaccines. If prepared from bacteria, they are correctly termed *bacterins*. The term "vaccine" is properly restricted solely to the immunizing agent against smallpox. However, it is widely used for any immunizing agent and is so used here.

Bacterins are so effective in preventing typhoid and paratyphoid fever that their use against these diseases in the United States military forces has long been a matter of regulation. The principle of the secondary stimulus is widely made use of in connection with these bacterins. After a person has received an ordinary course of three weekly inoculations as a primary stimulus, his resistance is maintained by single, annual, intradermal injections of 0.1 ml of

bacterin. These cause little or no reaction, are quick, inexpensive and easy and, above all, effective in reinforcing waning immunity.

The hypodermic injection of carefully controlled doses of suspensions of killed micrococci (staphylococci) is sometimes used to increase resistance of persons susceptible to boils, styes, etc., which are usually due to micrococci. The use of *Hemophilus pertussis* bacterins in preventing and modifying whooping cough is of great value also. As pointed out previously, these are often combined with toxoids.

(C) **Immunization with Attenuated, Living, Infectious Agents.** The third method of artificial active immunization consists in actually infecting the person or animal to be immunized with the desired organism which has been so treated that the virulence is greatly attenuated or diminished.

There are at least three means of lowering the virulence of pathogenic organisms so that they can be safely used to induce active, artificial immunity. These are (1) *animal passage*; (2) treatment with unfavorable agents, such as *desiccation*; and (3) *cultivation on special media or under special conditions* such as abnormally high temperature. An example of each will be described.

**ANIMAL PASSAGE.** This is well illustrated by the development of vaccine against fox distemper, a scourge to fox-fur farmers. The virus from a sick fox was injected into a ferret. Infectious fluid from the sick ferret was injected into another ferret. This ferret-to-ferret transfer (animal passage) was continued through a long series. The virus became adapted to, and enormously virulent for, ferrets. When fluid from the last ferret in a long series was tested in a fox, it had little or no virulence for the fox. However, the fox was afterward found to be completely immune to natural fox-distemper virus. The virus had become highly adapted to ferrets but *modified* or *attenuated* with respect to foxes. The ferret-passaged vaccine has saved fox farmers from enormous losses.

Another illustration of the effect of animal passage on virulence is seen in preparation of smallpox vaccine. It was originally thought that cows became infected with smallpox but developed only the relatively mild cowpox. Contact with the cow (animal passage) was supposed to have modified the virulence of the original smallpox virus. It was then called vaccinia\* virus. Vaccinia virus causes a mild infection in man.†

In 1798, Jenner, then a country doctor but later a famous British scientist, observed that many dairy workers, associated with cows having cowpox, did not succumb during epidemics of smallpox. Experimenting, he found that if some of the serum or lymph from the pustules on the udder of a cow with cowpox were scratched into the arm of a human being, a very mild disease (*vaccinia*) resulted, with the formation of a single, localized, poxlike lesion. This soon healed, leaving a distinctive scar. Smallpox never developed in persons after infection with cowpox. The person thus safely became immune to smallpox through infection with cowpox. The resistance to smallpox generally lasts 3 to 7 years.

Vaccinia virus, as used today, is prepared by scratching the virus into the shaved and disinfected skin of a calf. When the pustules are "ripe" the lymph

\* Vacca is the Latin word for cow.

† It may also be that the two viruses are distinct "species," but sufficiently closely related so that the one immunizes against the other.

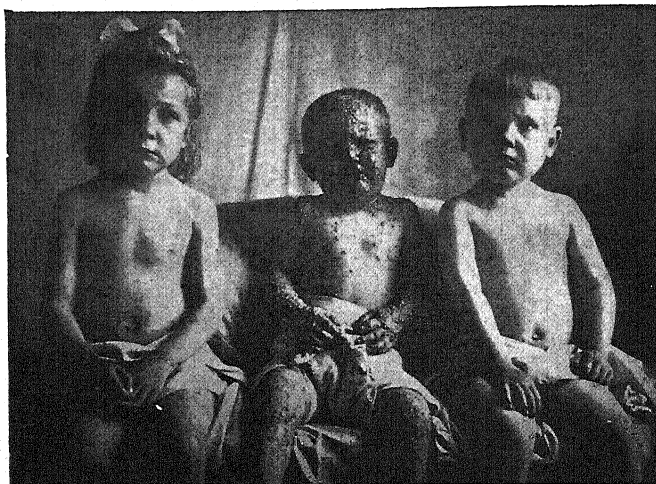


Fig. 22-2. Children of one family who were brought to the Municipal Hospital of Philadelphia with the mother and father, who had smallpox. The child in the center had been considered too young to be vaccinated. The other children had been vaccinated a year before; they remained free from the disease, although for several weeks they lived in the wards of patients with smallpox. (From Welch and Schamberg, "Contagious Diseases," Lea and Febiger, publishers.)

is collected from them and put up in glass tubes ready for use. Its potency and cleanliness are carefully controlled by the National Institutes of Health at Bethesda, Md. Its value is made evident by experience and by a glance at Figure 22-2.

"Modern instances" of the attenuation of virulence by animal passage are seen in the use of what is termed "avianized" rabies virus, and the preparation of yellow fever vaccine. As pointed out in Chapter 13, many microorganisms will multiply vigorously in living chick embryos. When rabies, yellow fever, smallpox and other viruses are thus "passed" from egg to egg, they lose much of their virulence for human beings and, while still capable of infecting mildly and thus immunizing, have lost the power of producing *severe* disease. The avianized rabies virus is widely used for the protection of dogs from rabies. The modified yellow fever virus (17D) has immunized millions of persons safely and effectively against this dread disease of certain tropical countries.

Modification of the virus of viral diarrhea of cattle by passage through rabbits likewise appears to furnish a very effective vaccine. Similar animal passage of hog-cholera virus, to produce a vaccine, is under investigation. Avianized canine-distemper vaccine is available; avianized smallpox vaccine will probably come into use. Studies on modified, live polio vaccine are being made by a number of investigators.

**DESICCATION.** Pasteur's name is immortalized in the term "Pasteur treatment" for rabies ("mad-dog bite"). Pasteur's process was not really a curative "treatment" but a course of immunizing injections with the living, attenuated virus of rabies.\*

\* In modern "Pasteur treatment" of human beings a killed virus is used.

If started soon enough after the bite of the infected animal, resistance (or interference) develops from the injections, before the virus from the infectious bite can cause disease.

For years Pasteur experimented with rabbits, dogs and guinea pigs (one of the many brilliant illustrations of the value of animal experimentation) until he felt certain that his method of attenuating rabies virus was safe and effective. It consisted in passing the virus from an infected dog to the brain of a rabbit and then from rabbit to rabbit (animal passage) to establish attenuated virulence for man. Like the fox-distemper virus which became very fatal for ferrets, the rabbit rabies virus acquired a maximum and fixed virulence for *rabbits*. Pasteur called this virus "virus fixé." The potency of the virus for *man* was further reduced by *desiccation*. Desiccated virus fixé was injected in increasing doses.

Pasteur's first human immunization against rabies, in 1885, was an extremely dramatic event and marked an epoch in the progress of the war on disease. Indeed, Pasteur is referred to as "the father of immunology." His experiments on rabies have been movingly described by Vallery-Radot, Pasteur's grandson.

CULTIVATION IN SPECIAL MEDIA. IMMUNIZATION AGAINST TUBERCULOSIS. A method for immunization against tuberculosis with living, attenuated tubercle bacilli has been known for some years. The procedure is known as the "Calmette-Guerin process" and the attenuated cultures as "BCG" (Bacillus Calmette-Guerin\*). The process consists of the injection of *live* tubercle bacilli, the virulence of which has been reduced by cultivation of the organisms on certain media containing bile. This method of cultivation induces the development of a stable variant of the tubercle bacillus having low virulence. This is the only important use, at present, of immunization of human beings with *living* bacteria. Its safety and effectiveness have frequently been questioned.

The W.H.O. † regards BCG vaccination as the most important means for the eradication of tuberculosis in countries *where other approaches to the problem are not practical*. The American Trudeau Society, in 1949, adopted appropriate recommendations regarding the use of BCG. It is felt by some that there is a tendency to rely too much on BCG, and as a result, to neglect other time-tested and sure means of control such as proper nutrition, x-ray surveys, hospitalization, sanitation, etc.

ATTENUATION BY CULTIVATION AT UNFAVORABLE TEMPERATURES. Although this method is not much used at present, its discovery and demonstration by Pasteur constitute an episode of great dramatic interest in the history of bacteriology. The principle was first used with *Pasteurella avicida*, the bacterial cause of fowl cholera. It was later developed in connection with studies of anthrax which, in the nineteenth century, decimated the sheep flocks of France and still does so in some parts of Europe. Following numerous preliminary experiments by the tireless Pasteur, on variants of *Bacillus anthracis*, cultures of the organisms were prepared by incubation at unfavorably high temperatures of 39° to 43° C (optimum around 35° C). This

\* Calmette and Guérin are the names of the two French scientists who developed the method.

† W.H.O. = World Health Organization, United Nations.



caused them to lose some of their virulence (and also, often, their power of spore formation).<sup>\*</sup> The immunizing agent finally adopted by Pasteur consisted of living, broth cultures of these attenuated strains. The first great public demonstration of the value of anthrax immunization gave the indomitable Pasteur some uncomfortable hours, because his ideas had been unfavorably regarded by many and his experiments were closely and critically watched. He injected about 100 sheep with his attenuated organisms. After a suitable period had been allowed for immunity to develop, these animals, as well as an equal number of normal, unprotected animals, were publicly inoculated with large doses of fully virulent anthrax bacilli. This experiment has been dramatized in a Hollywood motion picture. The test injections were made on May 31, 1881, at Pouilly le Fort. There were many skeptics in the audience when the injecting began.

A day or two later, when Pasteur arrived at Pouilly le Fort, "the carcasses of twenty-two unvaccinated sheep were lying side by side; two others were breathing their last; the last survivors of the sacrificed lot showed all the characteristic symptoms of splenic fever (anthrax). All the vaccinated sheep were in perfect health."<sup>†</sup>

## PASSIVE IMMUNITY

### ARTIFICIAL PASSIVE IMMUNITY

In some cases it is necessary that a large supply of antibodies appear in the blood immediately in order to combat an overwhelming infection. This is especially well illustrated in such diseases as diphtheria and tetanus. The chief symptoms in these diseases are caused by the bacterial toxins in the body of the patient. The poisonous action is very rapid. When the patient is already ill there is no time to lose waiting for him to develop active immunity, natural or artificial. He must passively receive ready-made antibodies. Immunity resulting from injections of these ready-made antibodies is called *passive immunity*.

It is now possible to purchase, at all well-stocked pharmacies and health departments, syringes or ampules already filled with antitoxic serum, prepared for just such emergencies. Such antibody-containing serum is obtained from animals, usually horses, which weeks or months previously have received repeated injections of the special antigen against which antibodies are desired.

**Passive Immunity in the Prevention of Disease.** Passive immunity is used in the prevention (*prophylaxis*) as well as in the cure of disease. For example, if it is known or suspected that a person is likely soon to become exposed or has very recently been exposed to certain diseases it is, under some circumstances (to be determined by the physician) an excellent plan to inject a small quantity of serum, or some derivative of serum (e.g., gamma globulin), containing the appropriate antibodies as a preventive or prophylactic measure. Diseases against which this form of prophylaxis is most commonly used are

<sup>\*</sup> The method has since been shown to vary in result so that neither loss of spores nor cultivation at 42° C should be assumed to have deprived these organisms of their dangerous properties.

<sup>†</sup> From "The Life of Louis Pasteur," by René Vallery-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.

measles, diphtheria and tetanus. Effective sera are available for some other infections (infectious hepatitis, rabies, pertussis) but are not so widely used. Some are still under investigation, like the serum (gamma globulin) for poliomyelitis.

**Passive Immunity and Serum Jaundice.** Attention has already been called to the fact that a considerable number of apparently normal and healthy persons carry, in their tissues and blood, the virus of *serum hepatitis* or *homologous serum jaundice*. It is a good example of a latent viral infection. Unless suitable precautions are taken to prevent, the virus is readily transmitted in the blood of donors, in gamma globulin, and in other blood derivatives. It is also easily transmitted by improperly sterilized syringes, needles, razors, and other objects carrying blood or serum or lymph from one person to another.

#### NATURAL PASSIVE IMMUNITY

Most antibodies can pass the placenta and consequently they are commonly found in the blood of the infant at birth. They serve to protect the child for some months after birth. After that the child becomes susceptible to many infectious diseases. It is therefore advisable to begin active immunization of the child early (second month to sixth month) against pertussis and diphtheria. Later, or simultaneously, tetanus toxoid may be given and still later immunization against polio and less common diseases if desired.

It is important to note that antibodies in the newborn infant imply immunity in the mother. The expectant mother who does not have good antibody titers to diphtheria, tetanus, pertussis and possibly salmonellosis and polio would do well to receive immunizing injections to confer passive immunity on her child.

**Transitory Nature of Passive Immunity.** The antibodies in all artificial passive immunity disappear from the body in two to three weeks, but while they are present they may entirely prevent an infection or greatly lessen its severity. In infants the maternal antibodies seem to persist for one to several months longer, probably because they are of human origin.

#### REFERENCES

- Brown, G. C.: Effect of booster inoculations on the serologic status of children vaccinated with poliomyelitis vaccine. *Am. J. Pub. Health*, 1955, 45:1401.
- Carpenter, P. I.: Immunology and Serology. W. B. Saunders Co., Philadelphia, 1956.
- Diphtheria and Pertussis Vaccination. World Health Organization Technical Report Series, No. 61, May, 1953.
- Editorial: Typhoid in vaccinated persons. *J.A.M.A.*, 1954, 154:1265.
- Edsall, G.: Immunization. *Ann. Rev. Microbiol.*, 1955, 9:347.
- Koprowski, H., and others: Immunization of infants with living, attenuated polio virus. *J.A.M.A.*, 1956, 162:1281.
- Paul, J. R.: Indications for vaccination against poliomyelitis. *J.A.M.A.*, 1956, 162:1585.
- Peck, F. B. Jr., Powell, H. M., and Culbertson, C. G.: Duck-embryo rabies vaccine. *J.A.M.A.*, 1956, 162:1373.
- Queries and Minor Notes: Tetanus immunization. *J.A.M.A.*, 1955, 157:1663.
- Raffel, S.: Immunity. Appleton-Century-Crofts, New York, 1953.
- Sabin, A. B.: Present status of attenuated live-virus poliomyelitis vaccine. *J.A.M.A.*, 1956, 162:1589.
- Sauer, L. W., and Tucker, W. H.: Immune response to diphtheria, tetanus, and pertussis antigens, aluminum phosphate adsorbed. *Am. J. Pub. Health*, 1954, 44:784.

- Strode, G. K., Editor: Yellow Fever. McGraw-Hill Book Co., New York, 1951.
- Vaccination Against Tuberculosis. World Health Organization Technical Report Series, No. 88, 1954. Columbia University Press, New York.
- Vallery-Radot, R.: The Life of Louis Pasteur. Doubleday-Doran & Co., New York, 1926.
- Various Authors on rabies control. Am. J. Pub. Health, 1955, 45:998, 1005.
- Various Contributors: Discussion of prospects for control of poliomyelitis. J.A.M.A., 1955, 158:1249, 1258, 1266, 1271, 1274.
- Volk, V. K., Top, F. H., and Bunney, W. E.: Observations on the effectiveness of various scarlet fever antigens in multiple antigen preparations. Am. J. Pub. Health, 1953, 43:833.
- Volk, V. K., Top, F. H., and Bunney, W. E.: Reinoculation with multiple antigen preparations of free-living children previously inoculated with multiple antigen preparations. Am. J. Pub. Health, 1953, 43:821.
- W. H. O.: Yellow Fever Vaccination, Monograph Ser. No. 30, 1956. Columbia Univ. Press, New York 27, N. Y.
- Yi-Yung Hsia, D., Lonsway, M., Jr., and Gillis, S. S.: Gamma globulin in the prevention of infectious hepatitis. New England J. Med., 1954, 250:417.

## Immunology and Microbiology

### 3. THE TISSUES IN RELATION TO IMMUNITY

#### THE ALLERGIC STATE

WE HAVE pointed out that tissue cells which have responded to an antigenic stimulus are immunologically different from tissue cells which have not so responded. The antigenically stimulated cells often retain antibodies in or on them for long periods of time. Under proper circumstances, when the specific antigen is again brought into contact with antibody-containing cells, the cells react in a very distinctive manner. They are said to be in a *hypersensitive* or *allergic*\* state. The particular tissues or organs involved in any allergic, antigen-antibody reaction are often called the *shock tissues* or *shock organs*.

**The Induction Period.** A period of several weeks must elapse between the first contact with antigen (the *sensitizing stimulus*) and that (the *toxic dose*) which causes the allergic reaction. This *induction period* allows time for antibody formation.

**Passive Allergy.** In some types of allergy normal tissue cells which have not had any active antigenic stimulus may *passively* acquire the antibodies necessary to an allergic reaction by merely adsorbing them from the blood stream. The antibodies which sensitize may be experimentally introduced into the blood of a normal animal by injecting serum from an immune animal.

Since, in such cases, the antibodies are passively received this mode of becoming allergic is often spoken of as *passive allergy*. It is analogous to passive immunity. In passive allergy the involved tissues are ready to react within an hour or so after they have adsorbed the antibodies.

**Allergens and Reagents.** In some common forms of allergy (represented by "hives" and the like) the antibodies involved appear to be precipitins or other familiar types of antibody. They are: (a) demonstrable in the serum and (b) readily transferred from one animal to another.

In other forms of allergy the antibodies are quite unlike any that we have yet described. They remain closely associated with the cells that produce them and are not readily demonstrable in the blood stream. Not much that is

\* From the Greek words *allos*, for changed, and *ergon* for activity.

definite is known of these antibodies. They are often spoken of as allergic *reagins*. The antigens that engender them also differ from substances commonly thought of as antigens, and are commonly called *allergens*.<sup>\*</sup> Examples of some of these allergens are sulfonamides and other drugs, certain cosmetics, certain dyes on fabrics, some plastics, antibiotics, etc. Hypersensitivity to these substances is commonplace.

While these allergens are not antigens as previously described, it is thought that they may become conjugated with proteins and other body components so that they act like complete antigens in such relationships.

### TYPES OF ALLERGIC REACTION

There are hundreds of different manifestations of allergy. The nature of an allergic reaction depends on many factors: the shock tissue; the nature of the allergen; the nature of the reagin; the anatomy of the animal involved (i.e., the location of the shock tissue); the dose of antigen; the degree of hypersensitiveness; and so on.

We may classify allergic reactions into two general types: (1) the *immediate wheal* or *anaphylactic* type, and (2) the *delayed* or *tuberculin* type. In general, the immediate type of allergy is produced by soluble-protein antigens, like egg-white or serum, while delayed allergy is evoked by cellular antigens such as bacterial cells. The types of allergy are not always easily differentiated. Sometimes both appear to be present together, and the same antigen may at times produce one and at other times another. General characteristics of each kind of reaction may be tabulated as follows:

#### I. Immediate Allergy

- (a) Visible reaction of sensitive tissues *occurs within seconds or minutes* after contact with antigen.
- (b) Is due to antibodies which circulate in the blood stream (notably precipitins) and which, therefore, are readily *transferable*, in serum, from one animal to another.
- (c) Tissues affected are primarily *smooth muscle, blood vessels and supporting tissues* like cartilage, fibrous tissue, etc.
- (d) *Desensitization* is relatively easy.

#### II. Delayed Allergy

- (a) Visible reaction of sensitive tissue occurs only hours following introduction of antigen and develops fully during one or more days.
- (b) Is due to antibodies (reagins) closely attached to, or inside of, cells, not readily demonstrable or transferable in serum. Transferable only in cells.
- (c) Any tissue may be affected.
- (d) Desensitization is difficult or impossible.

### MANIFESTATIONS OF IMMEDIATE ALLERGY

**Anaphylaxis.**<sup>†</sup> This is one of the best known, though still incompletely understood, manifestations of immediate allergy. The antibodies mainly involved appear to be precipitins and/or antibodies like precipitins. The anaphylactic reaction received its name because it was first thought to be a para-

<sup>\*</sup> Some workers use the terms reagin and allergen for *all* antibodies and antigens referred to in allergy.

<sup>†</sup> *ana* is from a Greek word meaning reverse; *phylaxis* is from a Greek word meaning protection.

doxical immune reaction *against immunity*. It is, however, no paradox but a sort of perversion or overreactivity of a truly protective mechanism.

Anaphylaxis results usually from antigen-antibody combination throughout the sensitized body following rapid, wide distribution of the specific antigen in blood and tissues. This rarely occurs in nature but is generally brought about by intravenous injection of considerable doses of antigen. It can also result from absorption of antigen from a subcutaneous hypodermic deposit or even from antigen inhaled (hay fever), ingested (food allergy) or absorbed through the skin (allergic dermatitis).

**THE ANAPHYLACTIC REACTION.** Within about five minutes after introduction of the toxic or shock dose of specific antigen, the animal (if a guinea pig) becomes uneasy and scratches at its nose, coughs, and is evidently embarrassed for air. Gagging movements occur, and the animal gasps for breath. Urination and defecation take place, the animal falls on its side and ceases to breathe. Death may supervene within a few minutes. If the attack is not fatal, recovery is often abrupt and seemingly complete within an hour or two and the animal will not exhibit hypersensitiveness for some days or weeks afterward. The animal is said to be *desensitized*.

Most of the symptoms are due to histamine-induced contraction of smooth muscle fibers, i.e., smooth muscle is the most obvious shock organ in anaphylaxis. Large amounts of smooth muscle are present in the lungs, thus constricting the air passages. The fibers are also present in the intestines and bladder. Reductions in temperature, swelling, inflammation, and other signs are probably due to the release of histamine or H-substance, by the tissues involved. Hence, antihistaminic drugs are of particular value in the immediate types of allergic reaction, represented by anaphylaxis. In rabbits, dogs, and other animals the picture varies, partly due to differences in anatomical location of smooth muscle. In pregnant animals abortion often occurs because the uterus consists largely of smooth muscle. In dogs there is much damage to the liver and this alters the clinical picture markedly in them. In immediate allergy there is also damage to blood vessels, resulting in escape of fluid into the tissues, causing swelling (*edema*). There is also decreased ability of the blood to coagulate (*hemophilia*).

#### OTHER REACTIONS OF IMMEDIATE ALLERGY

In human beings, typical anaphylaxis rarely occurs, but many other manifestations of immediate allergy are commonly seen. All are closely related in basic mechanism.

**Dermal Reactions. WHEEL-AND-FLARE.** A common manifestation of the immediate dermal type of allergic reaction is seen when a small amount of a specific antigen (e.g., horse dander) is introduced into the superficial layers of the skin of a person or animal whose tissues contain antibodies or reagins for that antigen. Within a few seconds or minutes there appears a hard, white swelling or *wheel* with irregular margins. This is quickly surrounded by an irregularly shaped, and often quite extensive, zone of redness called a "*flare*." These two signs (wheel-and-flare) are due to (a) edema; (b) dilation of the locally affected blood vessels. Both responses are characteristic of all types of immediate allergy, except the Arthus phenomenon, in which the vessels

contract. The whole allergic reaction itches intensely and usually disappears within a few hours.

"HIVES" (allergic urticaria) are closely related to this form of allergic manifestation. They often seem to result from the introduction of antigen into the skin by escape of some particular food substance, to which the patient is allergic, from the gastrointestinal tract into the blood stream. Hives may also occur in persons who have received doses of serum such as tetanus antitoxin for therapeutic purposes, and in other circumstances.

"HAY FEVER," ASTHMA, and the early stage of the COMMON COLD are other common manifestations of the immediate type of allergic reaction, occurring in ectodermal tissues in the respiratory tract.

All of the phenomena of the immediate allergic reactions appear to be due largely to histamine formed by the reacting cells. The response involves damage to, and dilatation of, blood vessels, edema, smooth-muscle contractions, and damage to supporting and connective tissues.

THE ARTHUS PHENOMENON. This reaction, first described by Arthus in 1903, depends on precipitins developed by repeated *subcutaneous* or *intra-dermal* injections of an appropriate antigen (foreign serum, egg white, etc.). As more precipitins develop, the antigen-antibody reactions in the tissues at the sites of the repeated injections become progressively more and more violent. In well-marked Arthus reactions prolonged smooth-muscle contractions occur in the walls of the local blood vessels, and damage to the vessel walls develops.

Local edema and erythema occur, as in most allergic reactions. The contracted blood vessels become occluded with clots and the area, deprived of blood supply, becomes necrotic (dead and disintegrating). *This reaction will not occur in tissues without blood vessels.*

#### MANIFESTATIONS OF DELAYED ALLERGY

The response in delayed allergy appears many hours, even several days, after contact between sensitive cell and allergen. The reaction involves a local inflammation and sometimes, with large doses of allergen, local necrosis. Histamine is *not* involved. One of the best known examples of delayed allergy is the *tuberculin reaction* (Chapt. 30), a form of bacterial allergy.

**Bacterial Allergy.** Bacteria, as well as viruses and other microorganisms entering the blood stream or tissues, sensitize certain cells of the body as well as stimulate the production of familiar types of antibody. In many instances the tissues of the skin seem to become especially sensitive, a condition readily demonstrated by intradermal injection of the antigen (allergen), which results in a local redness and swelling. This is due to the presence of the specific antibodies (reagins) in the cells of the skin. The allergin-reagin reaction occurs at once but the result is not manifest for several hours or days.

**Allergy and Disease.** Whenever an infectious disease becomes subacute or chronic, body cells may become sensitized to one or more of the microbial antigens. The sensitivity may be purely of the delayed type or both immediate and delayed sensitivity may be present in varying degrees. These situations give rise to varying symptoms and little-understood reactions. A person who has been infected with tubercle bacilli remains for months or years in an allergic condition to the organism, an important consideration in regard to

his resistance to the disease. As previously pointed out, BCG vaccination against tuberculosis is done with living tubercle bacilli. The resulting allergy against the organisms, as manifested by development of a positive tuberculin reaction, is believed to be the basis of resistance to the disease. Skin tests for allergy to tubercle bacilli (called *tuberculin tests*) are of great value in the study of tuberculosis (see Chapter 30).

#### HARMFUL EFFECTS OF ALLERGY

Like many normal and beneficent physiological functions, allergic reactions may at times be so violent *in some persons* as to be harmful. These few violent reactions, like violent acts, create more comment and attract more attention than the enormous number of normal and helpful reactions which go on constantly unnoticed. For example, certain persons appear to become excessively allergic to hemolytic streptococci. The heart and joints appear to be "shock tissue" in such allergy. There is believed to be a close relation between this allergy to hemolytic streptococci and rheumatic heart disease, one of the most important causes of disability and death in the United States. Many chronic, disabling conditions, especially forms of asthma and joint disease, are thought to be related to allergic reactions of certain tissues to obscure microbial infections such as chronic sinusitis. Allergy, therefore, plays an important part in chronic infectious diseases. Other diseases of this chronic nature are syphilis, undulant fever, swine erysipelas and leprosy. Many of the rashes and eruptions seen in bacterial and viral diseases are allergic reactions of the delayed type.

#### ALLERGY AS A DEFENSIVE MECHANISM

Once the body has been subjected to an antigenic stimulus, the more superficial tissues\* acquire a greatly enhanced power to bind and localize the specific antigen when later brought into contact with it. The effectiveness of *tissue immunity* is thus greatly enhanced by allergy, as are antibody and phagocytic defense also. The Arthus phenomenon, and dermal reactions generally, are illustrations of this heightened reactivity. The antigen thus bound, although perhaps doing considerable damage locally where bound, cannot spread throughout the body causing injury to deeper and more vital tissues.

**Non-antitoxic Immunity to Toxin.** An example of this is seen when rabbits and guinea pigs are made allergic by injections of proteins extracted from diphtheria bacilli. The animals are able to survive doses of live, toxigenic diphtheria bacilli which always kill normal animals. The toxin is held and bound in the skin. While it does severe damage locally, it cannot spread to the deep, vital tissues and so the animal survives easily *in the complete absence of antitoxin*.

**The Koch phenomenon** is another classical example of the binding power of tissues. If a normal guinea pig is inoculated, in the right groin, with virulent tubercle bacilli, the bacteria gain a foothold, form a local abscess, and then proceed, almost unopposed, from the abscess to the lymph nodes of the abdominal cavity, to the spleen, the liver, the lymph nodes of the thorax, the

\* Especially cutaneous tissues, and those lymph systems which drain tissues in contact with the exterior, such as respiratory and gastrointestinal tracts.



lungs and kidneys, and the pig dies of disseminated tuberculosis in six to eight weeks. Now, if, on the second or third week of this progressive disease, a second injection of tubercle bacilli is made into the left groin, there is a strong, local, allergic, tissue reaction. The bacilli are *closely held* in the site where they are injected and *do not progress further*, although they may cause a local abscess. The tissues are highly defensive because of the allergy to the first infection.

The guinea pigs are so susceptible to tubercle bacilli that they eventually die in spite of the valiant defense put up by the tissues. Human beings are, in general, much more resistant. Most adult human beings have had a mild, unrecognized, and long-since-healed infection with tubercle bacilli. Because of this they are much more resistant to tuberculosis than persons who have never had any contact with tubercle bacilli. The first group are allergic (hence resistant) to the bacilli, as shown by the fact that they react to tuberculin. The second group may be made allergic (resistant) to tubercle bacilli by giving them a very mild infection as is done in BCG vaccination. Allergy is thus revealed as a potent defensive mechanism.

#### REFERENCES

- Banic, S.: Entre l'hypersensibilité et l'immunité. *Revue d'Immunologie*, 1953, 17:135.  
Berger, A.J.: Sensitivity reactions. *Am. J. Nursing*, 1955, 55:948.  
Brown, E. A.: Problems of drug allergy. *J.A.M.A.*, 1955, 157:814.  
Chase, M. W.: The Allergic State. In Dubos, R. J., *Bacterial and Mycotic Infections of Man*. 2nd ed. J. B. Lippincott Co., Philadelphia, 1952.  
Editorial: Diagnostic value of skin testing. *J.A.M.A.*, 1955, 157:825.  
Frobisher, M., Jr., and Parsons, E. I.: Studies on type specific immunization with somatic antigens of *Corynebacterium diphtheriae*. *Am. J. Hyg.*, 1950, 52:239.  
Kahn, R. L.: Tissue response in immunity. *Ann. New York Acad. Sci.*, 1955, 59(Art. 3):281.  
Katz, S., and Dingle, J. H.: Antihistamines and the common cold. *Am. J. Nursing*, 1954, 54:179.  
Lautrop, H.: On the existence of an antibacterial factor in diphtheria immunity. *Acta Path. et Micr. Scandinavica*, 1955, 36:274.  
Rinkel, M. J., Randolph, T. G., and Yeller, M.: *Food Allergy*. Charles C Thomas, Springfield, Ill., 1951.  
Rostenberg, A., and Webster, J. R.: Mechanisms of cutaneous drug reactions, especially to antibiotics. *J.A.M.A.*, 1954, 154:221.  
Warwick, W. J., Page, A., and Good, R. A.: Passive transfer with circulating leukocytes of delayed hypersensitivity to cat scratch antigen. *Proc. Soc. Exp. Biol. & Med.*, 1956, 93:253.

## Microorganisms and Disease

**Obstacles to Parasitism.** Most microorganisms of the outside world lack the physiological properties which might enable them to multiply within a plant or animal host.\* If we consider mammals as the host, many microorganisms of the outer environment find the body temperature too high. Others find it too low. Even though the temperature may be suitable, many cannot live in contact with organic material. Others require cellulose as a source of carbon. Still others require a different pH, or do not find suitable nutrient, and so on. Some find conditions in or on the animal body which will permit their growth, but they cannot resist the action of the phagocytes.

The first requisite to successful parasitism is, then, ability to live *in* or *on* the host, and this without stirring up an antibody, phagocytic or allergic reaction with which the parasite cannot cope.

**What Is Disease?** An exact definition of disease is difficult to formulate. We may say that disease is a departure from normal. But what is normal, and how great must be the departure before it falls into the category of disease? Possibly the simplest plan for the present is to fall back on the generally accepted meaning of the term disease and leave such academic arguments to the lexicographers. Disease, we shall say, is any visible or sensible, harmful departure from the "normal" condition of the body. Such conditions often result from the entrance into the plant or animal body, of organisms which are able to multiply in (or on), and/or to damage, body tissues. Such disease-producing (pathogenic†) organisms are often classed as *parasites*. They may be macroscopic (e.g., various insects and worms) or microscopic (e.g., bacteria, yeasts and molds, rickettsiae, PPLO, viruses and protozoa). As we shall see later, certain true saprophytes can be very pathogenic, but these are rather the exception to the rule.

**Parasitism and Pathogenicity.** It is sometimes difficult to distinguish between parasitism and pathogenicity. A parasite is an organism which lives at the expense, and upon the substance of the host, giving nothing in return. It may or may not be significantly pathogenic. For example, a mosquito might be regarded as a harmless parasite. But if its bite causes a severe allergic reaction, then it is a pathogen.

\* Host, in this sense, is an infected or infectible plant or animal.

† *patho* is from the Greek root *pathos*, meaning sickness.

**Pathogenic Saprophytes.** Pathogenicity does not always involve parasitism. For example, certain organisms like the bacilli causing lockjaw (*Clostridium tetani*) cannot invade or live in normal tissues and are in no sense parasites. They can live only in dead (necrotic) material, such as might occur in a crushed foot. They are true saprophytes. They can cause fatal disease because they give off a toxin which is absorbed from the site of their growth.

Similarly, the organism of food poisoning (*Cl. botulinum*) cannot, as a rule, multiply in the tissues or on the body, but produces a poison in foods outside the body. When such food is eaten the preformed toxin produces a highly fatal disease.

Such organisms are *pathogenic saprophytes*.

**Pathogenicity Is Fortuitous.** Those not familiar with microbial disease sometimes conceive of microorganisms as predatory, purposeful creatures producing poisons and invading the tissues for the sole purpose of causing harm as though that were to their benefit in the sense that a tiger, snake or spider benefits from killing its prey. Microorganisms are sometimes thought of as being endowed with special powers for injury. Like dandelions in the lawn, microbial growth occurs only where (and because) conditions permit. They have no evil intent. Their only interest is propagation of the dandelion or the microbe. If the lawn-owner or host reacts against them with 2-4D or antibiotic it is only because he is not yet adapted to them. *Some* people like the pretty flowers or make delicious salads and wine out of dandelions! However, excessive irritation to the host by plant or microbe results unfavorably to the parasite. The greatest advantage to the parasite results from unnoticed parasitism, not from cannibalism!

**Mutual Adaptation.** The process of adaptation between host and parasite must be thought of as a mutual one, requiring many generations of natural selection of the most compatible. Microbial generations are sometimes very short, a few minutes or hours; human generations are long, about 25 years. Thus, microorganisms can become adapted to growth in contact with human tissues before man acquires species or racial resistance to invasive microorganisms.

We may imagine that a few species of microorganisms are just beginning the process of adaptation, since they stir up a violent reaction (acute disease) nearly every time they come into contact with the host and begin multiplying. Gonococci and measles virus are examples. The human host has not yet become adapted to them. Other parasites are better adapted; the host, through many generations of contact, seems to have become more used to having them growing within him, and chronic disease ensues. Syphilis, tuberculosis and leprosy may be thought of as examples. In still other instances an individual human host and dangerous pathogens like the typhoid bacillus, hemolytic streptococci, pneumococci and meningococci rapidly become mutually adapted, with or without perceptible disease. They may live together for years with no evidence of disease. This is a very common situation and will be discussed later as the *carrier state*.

Very frequently the apparently highly adapted relationships represent in reality only a temporary armistice or armed truce; a microbiological "cold war." If one side weakens, the other automatically takes advantage of the situation. The important point here is that we are constantly in contact with

microorganisms capable of making us very ill or killing us. That they do not do so is because we have ample defensive mechanisms. Let these become weakened or breached, and disease, great or small, results.

### FACTORS IN THE OCCURRENCE OF DISEASE

In discussing the occurrence of infectious disease we have to consider several factors: (1) the means by which the parasite enters the body (*portal of entry*); (2) its means of maintaining itself there and multiplying (*vegetative vigor* or *aggressiveness*); (3) its *toxicity*; (4) the *defensive mechanisms* of the host; (5) the portal by which the parasite leaves one host to reach another (*portal of exit*); (6) the factors which affect survival of the parasite outside the host (*resistance of parasite to external environment*); (7) the mechanisms which effect transmission from one host to another (*vectors of disease*); and (8) *dosage*, or numbers of infecting organisms. Most of these factors are discussed in other chapters. The first, second, third and eighth are discussed here.

1. **Portal of Entry.** The portal by which an organism enters the body is important in determining the occurrence and kind of disease. As we have seen in Chapter 21, if the skin is kept intact, no ordinary microorganism can get through it.\* But if any slight cut or scratch exists, then microorganisms can get into the tissues. The thin membranes about the eye (*conjunctivae*), in the nose, throat, and lungs and in the genitalia are less able to withstand invasion of some microorganisms than the tough outer skin, and certain infections readily begin in such situations.

Certain microorganisms, under ordinary circumstances, can gain a foothold in the body only when they come into contact with the respiratory tract, others only through contact with the intestinal lining, and so on. Thus, dysentery bacilli (*Shigella*) rubbed over the hands, or even into a wound, would cause no infection while, if swallowed, they might produce a fatal disease. On the other hand, *Neisseria gonorrhoeae* might be swallowed without harmful effect but if rubbed into the eye or genitalia could cause gonorrheal infection of the mucous membranes.

The skin and many mucous membranes of the body are inhabited by large numbers of bacteria, some of them highly pathogenic if they get through the skin or membranes. They are usually held in check by the thick layers of epithelial (external covering) cells, phagocytic cells and by the binding powers of the local tissues.

2. **Vegetative Vigor (or Aggressiveness).** After having gained access to the body, saprophytic microorganisms, if not destroyed by acids or other injurious substances and if the temperature is not unfavorable, are usually detained in the tissues and/or immediately ingested and destroyed by leukocytes or destroyed by certain enzymes (e.g., lysozyme, pepsin, trypsin). Pathogenic microorganisms, however, frequently excrete poisons and enzyme-like substances which kill and drive away leukocytes. Such substances are called *leukocidins* and *antiopsonins*, respectively. There may at first contact be little or no binding power of local tissues. If the microorganisms avoid or destroy

\* Some few microorganisms are said to be able to penetrate the intact skin; probably they get into hair follicles and sweat glands.

the leukocytes and other tissue cells, they may have an opportunity to grow in the body.

As the microorganisms grow, they become more adapted to the host, and better able to multiply within it. Host-resistant mutants undoubtedly occur and are selectively favored by conditions in the host. For example, they often develop capsules which protect them from phagocytosis and antibodies. They can then grow into the lymph spaces, spreading widely through the tissues. Some however, as the diphtheria organism, remain entirely localized on surfaces, killing tissues only locally and, unfortunately for both host and parasite, excreting toxin which is adsorbed by the blood. Others may grow in the blood stream and then we have the condition called *viremia* or *bacteriemia*, or *rickettsiemia*; *septicemia* or "blood poisoning."

Having gained entrance by a favorable portal (i.e., a tissue favorable to their growth or maintenance\*), they may be carried by the blood (or even by leukocytes) and localize at various other points in the body, e.g., liver, spleen, bone marrow or lymph nodules of the intestine. Secondary abscesses (secondary foci of infection or *metastatic infections*) result.

**3. Toxicity.** Toxins of microorganisms are poisonous substances, mainly proteins or protein-like. Some microbial toxins appear to be polysaccharides. Others are hydrolytic enzymes.

The toxins of bacteria are of two sorts: *exotoxins* and *endotoxins*.

*Exotoxins* are poisonous waste products excreted by the bacteria into their environment. Exotoxins characteristically stimulate the production of antitoxins when injected into animals. Diphtheria toxin is a classical example of an exotoxin. Some exotoxins, such as those of *Clostridium botulinum* and certain micrococci, are harmful only when swallowed.† Others, like that of diphtheria bacilli, can be taken by mouth with impunity, but if injected or adsorbed into the blood even in very tiny doses, may cause death. Most exotoxins appear to have an affinity for nervous tissue and often for heart muscle, kidney, etc. They cause damage principally to those tissues. Most of these toxins are much more potent than cobra venom.

Some organisms produce exotoxins which kill leukocytes. The leukocidins of micrococci and streptococci are good examples. Others produce toxins which kill and destroy the cells of defensive tissues which tend to develop around foci of infection, forming retaining sacs. This seems to occur in progressive diseases like tuberculosis, actinomycosis and syphilis.

**ENZYMES AS TOXINS.** Some toxins are enzymes or are related to enzymes. For example, virulent streptococci, such as those causing scarlet fever, erysipelas and "blood-poisoning" (septicemia) produce an enzyme-activator called *streptokinase* or *fibrinolysin*. This helps to digest the fibrin in blood clots and this presumably enables the organism producing the enzyme to enter the blood stream and invade the local tissues (as in erysipelas). Many organisms also produce potent lipolytic (fat or lipid-digesting) enzymes and trypsinlike proteolytic enzymes. *Catalase*, an enzyme found in many species of microorganism and common in animal tissues, catalyzes the decomposition of hydrogen peroxide into water and oxygen. In several bacterial species,

\* This has sometimes been called the "tissue of predilection."

† Except under abnormal, experimental administration.

notably *Mycobacterium tuberculosis* and *Pasteurella pestis* (cause of bubonic plague), catalase production seems to be definitely associated with pathogenicity. *Lecithinase* is an enzyme which helps destroy erythrocytes. *Hyaluronidase* destroys hyaluronic acid, a clear, gummy, intercellular substance which normally opposes progress of microorganisms through the tissues. This enzyme is often spoken of as a "spreading factor."

There are many factors besides microbial exotoxins which determine whether infection will occur. Many are still very obscure. For example, organisms generally regarded as *perfectly harmless* saprophytes are sometimes found as the causative agent in a serious or fatal infection. Is this because of a defect in host defenses, or a sudden mutation of the organism to host-resistance and aggressiveness, or both?

ENDOTOXINS remain within the bacterial cell or are closely attached to it. Many endotoxins are the cell protoplasm itself. Like exotoxins, most bacterial endotoxins are proteins or substances associated with proteins. However, some important endotoxins are carbohydrates of the capsule, as in pneumococci and type B influenza bacilli. Just how they act in the body is not clear.

**4. Dosage.** This factor in establishing infection is a simple quantitative one, yet it also involves other factors. As a generality we may say that, under ordinary circumstances, the larger the dose of infective microorganisms the greater the chance that an infection will result. However, certain qualifications are necessary. For example, very large numbers of some organisms may be present in certain situations without causing any difficulty at all. The intestine contains thousands of billions of deadly bacteria at all times. Yet if only a dozen or so of some of these are introduced directly into the blood stream or injected into the brain, they can quickly set up a fatal infection. Similarly, one might swallow 3 or 4 typhoid bacilli with impunity yet several hundred might overcome local resistance and cause typhoid fever. With some organisms a single cell or particle is sufficient invariably to infect. Obviously much depends on the virulence of the particular organism involved and on the resistance of the tissues which it contacts, as well as on dosage alone.

**Infectiousness, Pathogenicity and Virulence.** Organisms which can (1) be readily transmitted from one host to another and (2) maintain themselves in a high percentage of the hosts which they contact may be said to be highly *infectious*. They may cause much or little damage and thus be of high or low *pathogenicity*. If they have little vegetative vigor and little toxigenicity they may have little *virulence*. They may have high *virulence* and great infectiousness yet in certain hosts cause little disease (low pathogenicity). For example, yellow fever and poliomyelitis have been so widespread in some communities that virtually all of the children under ten became infected and developed demonstrable antibodies yet few showed any perceptible evidence of disease at all. Most adults are immune to poliomyelitis yet never had a recognized case. Obviously there were many infections yet little disease. Thus, distinctions should be made when speaking of infectiousness, pathogenicity and virulence of microorganisms. They are commonly confused with each other. Everything is relative and dependent on something else.

**Koch's Postulates.** It is not always possible to be certain that the microorganisms isolated from a given disease lesion or from pus, blood or feces,

are the cause of the observed disease condition. Many harmless microorganisms are found growing in feces, sputum and ulcerating wounds. Some would not grow there unless the diseased condition existed first. Such adventitious organisms are called *secondary invaders*. To prove that a certain microorganism is the cause of a given disease often requires the most painstaking and careful study.

The question as to the etiological relationship of various bacteria to specific diseases was a very live one long before the time of Koch and there was much loose discussion and profitless argument regarding many bacteria and their relation to disease. When Koch established the pure-culture technique it became possible to apply exact methods to the study of the etiology of disease. He was very conservative in stating the relationships of any given organism to any particular disease.

His ideas on the subject were crystallized largely by his studies of the relationship of tubercle bacilli (*Mycobacterium tuberculosis*) to tuberculosis. Koch, like others before him, observed the bacilli in the lesions of animals dead of the disease. But he was not too ready to believe that he had discovered the cause of tuberculosis because he found certain organisms present in the lesions of tuberculosis. Might not this bacillus appear in the tissues merely accidentally because the animal, being so ill, is too weak to resist its invasion? Might it not be merely a relatively harmless opportunist? Might it not represent contamination with a common saprophyte capable of living in the necrotic tissue? Koch, involved in a discussion of the problem, finally stated what he believed to be the evidence necessary to prove an organism to be the cause of a disease. The evidence consists of four postulates, generally called *Koch's postulates* today, and they are, essentially, as follows:

1. The organism must be associated with all cases of a given disease and in logical pathological relationship to the disease and its symptoms and lesions.

2. It must be isolated from victims of the disease in pure culture.

3. When the pure culture is inoculated into susceptible animals or man, it must reproduce the disease (or specific antibodies thereto). Many such inoculations into man have been made on courageous volunteers. In others, accidental infections have occurred which have provided long-wanted evidence. The value of animal experimentation is here very evident.

4. It must be isolated in pure culture from such experimental infections.

Even today the etiological relationship of some bacteria to diseases which they are thought to cause has not been established firmly on the basis of Koch's postulates. A notable example is the relation of so-called *Mycobacterium leprae* to leprosy.

**Rivers' Postulates in Viral Diseases.** Viruses were unknown at the time of Koch's major works, so that he failed to take these invisible, noncultivable agents of disease into consideration in stating the criteria by which the causal relationship of a pathogen to a disease might be determined. Rivers, in 1937, outlined criteria similar to Koch's postulates, which might apply in the cases of viruses. Essentially these are as follows:

1. The virus must be present in the host cells showing the specific lesions, at the time of the disease, or in the blood or other body fluids.

2. Filtrates of the infectious material (blood, etc., or tissue triturates)

shown *not to contain bacteria or other visible or cultivable organisms*, must produce the disease or its counterpart in appropriate animals or plants.

3. Similar filtrates from such animals or plants must transmit the disease.

#### REFERENCES

- Bauer, T. J.: The communicable disease problem in the United States. *J.A.M.A.*, 1955, 158:1407.
- Burnet, Sir Macfarlane: *The Natural History of Infectious Disease*. Cambridge Univ. Press, New York, 1953.
- Dubos, R. J.: *Biochemical Determinants of Microbial Disease*. Harvard Univ. Press, Cambridge, Mass., 1954.
- Dubos, R. J.: Second thoughts on the germ theory. *Sci. Am.*, 1955, 192:31.
- Howie, J. W., and O'Hea, A. J., Editors: *Mechanisms of Microbial Pathogenicity*, 5th Symposium, Soc. for Gen. Microbiol., Cambridge Univ. Press, New York, 1955.
- MacLeod, C. M., and Pappenheimer, A. M., Jr.: Properties of Bacteria Which Enable Them to Cause Disease. *In* *Bacterial and Mycotic Infections of Man*, 2nd ed. J. B. Lippincott, Philadelphia, 1952.
- Nungester, W. J., Moore, V., Mika, L., and Summers, P. W.: Comparative study of host resistance of guinea pigs and rats. *J. Inf. Dis.*, 1955, 96:227.
- Oakley, C. L.: Bacterial toxins. *Ann. Rev. Microbiol.*, 1954, 8:411.
- Wedberg, S. E.: *Microbes and You*. The Macmillan Co., New York, 1954.
- Wilson, G. S., and Miles, A. A.: *Principles of Bacteriology and Immunity*. 4th ed. Williams & Wilkins Co., Baltimore, Md., 1955.



## Transmission of Disease

### VECTORS

MANY MICROORGANISMS have some degree of self-mobility in fluids. It is generally limited, however, to distances of the order of a few inches or feet. They cannot travel or swim long distances, or fly or climb, of their own volition. However, they may be carried many miles in rivers and lakes, or by aerial or marine currents. Indeed, most microorganisms depend on various extraneous moving agents for long-distance transportation: dust; water; food; droplets of saliva from sneezing and coughing; saliva-contaminated hands; articles contaminated with oral, nasal and/or intestinal matter; insects; animals. All of these are important *vectors* (transmitting agents) of disease.

While pathogens of plants and insects are usually fairly durable, a great difficulty in travel for many pathogens of mammals is that conditions in the world outside the body are too harsh. This is a penalty of extreme adaptation. Drying is fatal to some such as meningococci, gonococci and syphilis spirochetes. Exposure to sunlight quickly kills many mammalian pathogens such as tubercle bacilli. Others cannot live in natural bodies of water or in soil or in feces.

Most of the infectious diseases of man are produced by organisms which do not form heat-resistant spores. For this reason, with few exceptions, non-sporeforming pathogenic microorganisms are easily killed by boiling and by disinfectants like cresol and the halogens.

Furthermore, not only are infectious agents much restricted in the modes of travel available to them, but they must find a suitable portal of entry into a host at the end of their journey. The host must, in addition, be a susceptible individual. Nevertheless, in spite of their difficulties, like the lowly bedbug\* microorganisms get there just the same.

### TYPES OF DISEASE VECTOR

There are two general types of disease transmission: mechanical and biological. Each type of transmission may be accomplished in one or more of several ways. For convenience we may list these as follows:

- \* The moth has wings of velvet;  
The butterfly, wings of flame.  
The bedbug has no wings at all,  
But he gets there just the same! (*Old rhyme.*)

## 1. Mechanical by:

- (a) Fomites\*
- (b) Foods, including milk and water
- (c) Hands
- (d) Droplets of saliva or nasal secretion
- (e) Dust, infected from any source
- (f) Direct contact, as in the venereal diseases, or in nursing patients with discharging wounds, ulcers, etc.
- (g) Carriers†

## 2. Biological by:

- (a) Human blood and blood derivatives
- (b) Bites of animals
- (c) Bites of arthropods‡
- (d) Feces of biting or coprophagous§ arthropods
- (e) Bodies, feet, etc., of arthropods

It will be seen that the different modes of transmission are not necessarily mutually exclusive. Many diseases may be transmitted by several of these methods; others are restricted, under natural conditions, to only one or two methods.

## 1. MECHANICAL TRANSMISSION

(a) **Fomites.** It is obvious that any object having live, infectious microorganisms upon it may serve to transfer the bacteria from one person or place to another. Thus, soiled bed linen or clothing, eating utensils, toys, pencils and similar objects are dangerous after having been used by persons harboring microorganisms causing diseases of the intestinal tract (e.g., poliomyelitis, typhoid fever or dysentery), of the skin (e.g., smallpox, measles, boils, fungus infections), or the respiratory tract (e.g., diphtheria, scarlet fever, measles, influenza, pneumonia). Venereal diseases may be transmitted by some of these objects, although this is rare. Public toilet seats, drinking cups, hair brushes, etc., are always to be avoided for esthetic as well as sanitary reasons.

**SANITIZATION OF EATING UTENSILS.** The whole problem of sanitization of public eating utensils and eating places is receiving public discussion and thorough scientific study by progressive health departments and agencies. Problems involved are: methods of dishwashing; detergents, their properties and performance; effectiveness of disinfectants; tests for concentration of disinfectants in wash water; education of persons responsible for sanitation, and of the public; methods of examining utensils bacteriologically and so on.

Restaurant proprietors who have the well-being of their patrons in mind either carefully scald all dishes after washing them (Fig. 25-1) or, after thoroughly washing them in *hot* water with a good detergent, rinse them in clean, cool water containing at least 100 parts per million of available chlorine,

\* Fomites are objects contaminated by persons harboring infectious organisms; for example: hand hangers in buses, doorknobs, money, dishes, pencils, handkerchiefs, clothing, toys, bed linen, etc.

† Carriers are persons, often more or less recent convalescents from infectious diseases, who, though in apparently good health, nevertheless still harbor the infectious organisms and distribute them in their environment.

‡ Arthropods are invertebrates with jointed legs and feet. The Phylum Arthropoda includes crustacea (crabs, shrimps, etc.) and the groups, commonly called "insects," comprising flies, mosquitoes, ticks, lice, etc.

§ Feces-eating.

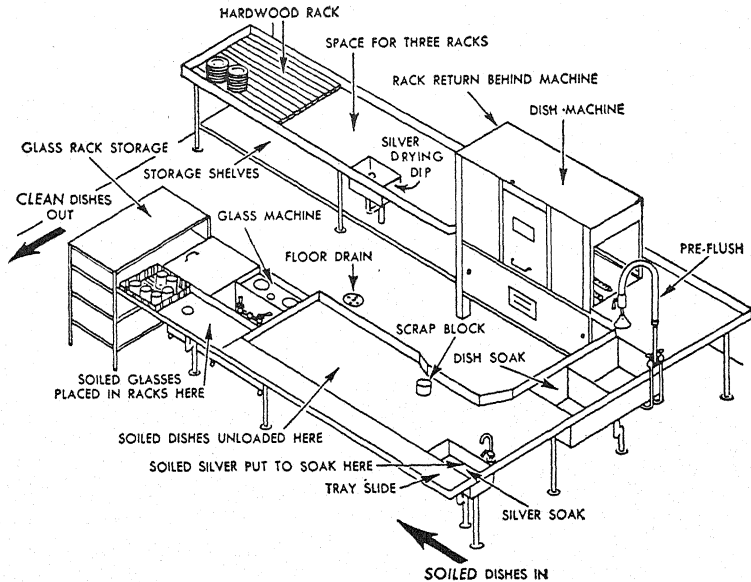


Fig. 25-1. One form of modern, sanitary, dishwashing equipment. The working bench is of stainless steel. Soiled dishes are piled on the bench in the foreground. They are sorted and scraped, the larger scraps of food dropping into a barrel beneath the counter. Glasses are rinsed over rotating brushes, dipped in disinfectant and placed in trays in a rack (left). Silverware soaks in a pan of special detergent solution (right foreground). The dishes, arranged in baskets, are soaked and then given a preliminary rinse with *hard* streams of *hot* water (right). They then pass through a machine dishwasher (center, background). The silver, after soaking, passes through the same process as the dishes and is self-dried after a dip into a drying agent. Afterward all utensils are stacked and stored in dust-proof cabinets. Eating utensils handled in this way are virtually sterile. (Courtesy, John L. Wilson and Wm. M. Podas, Economics Lab'y, Inc., in *Modern Sanitation*, May, 1950.)

and dry by drainage. The odor of chlorine around a lunch counter is a favorable sign. Other disinfectants, without taste or odor, are also widely used.

**EXAMINATION OF UTENSILS.** Methods for measuring and controlling the amount of bacterial contamination of dishes, etc., are not yet exact. Many technical problems are involved and are under investigation. Most of the methods at present favored for bacteriological examination of eating utensils center around some modification of the *swab-rinse technique* which involves the use of a sterile cotton swab made on a wooden applicator. In a simple procedure the swab is moistened in a bland collecting fluid (water, broth or buffered saline solution) and used to wipe a certain prescribed area of the utensils within a few hours after they have been washed and dried. The swab is then shaken thoroughly in a known volume (10 ml) of sterile saline solution, or better, broth, in a vial.

After shaking the swab in the collecting fluid, dilution-plate counts are made of the bacteria in the fluid. From the numbers of colonies obtained an estimate is made of the degree of contamination on the dishes, etc. Commonly, a minimum standard of 100 organisms per utensil is recommended.

**SOLUBLE SWABBING MATERIALS.** Many of the bacteria collected from eat-

ing utensils or other objects with cotton swabs are not released by the cotton fibers to the collecting fluid in which the swab is shaken, even with violent agitation. A special, soluble swab material, such as calcium alginate (fibers composed of a buffer-soluble, vegetable-gum salt), releases all of the attached bacteria by dissolving in the collecting fluid.

STICKY AGAR SURFACES have also been used to remove bacteria from eating utensils, and may be preferable to swabs.

STANDARD PROCEDURES. Methods of washing and handling utensils, time and temperature of storage, humidity, dust, insects and so on, are factors affecting the numbers of organisms surviving on the utensils and dishes. A standardized method, taking these and other factors into consideration, has been outlined by a Committee of the American Public Health Association. This specifies formulae for media, solutions, area swabbed, method of swabbing, cultural details, etc. There are also other, direct methods (Fig. 25-2).

Unfortunately, there is no exact method of measuring the amount of disease spread by dirty dishes and eating utensils in unhygienic restaurants. We must all have a good deal of immunity to the microorganisms so transmitted. There is, however, much indirect evidence that unsanitary eating utensils are responsible for a great deal of disease transmission. From an esthetic viewpoint, one does not like to feel that a little saliva from previous patrons is being included, gratis, with his meal.

PAPER DISHES. The use of paper cups, dishes and eating utensils is the best step toward eliminating the sanitary evils of public glass and chinaware and

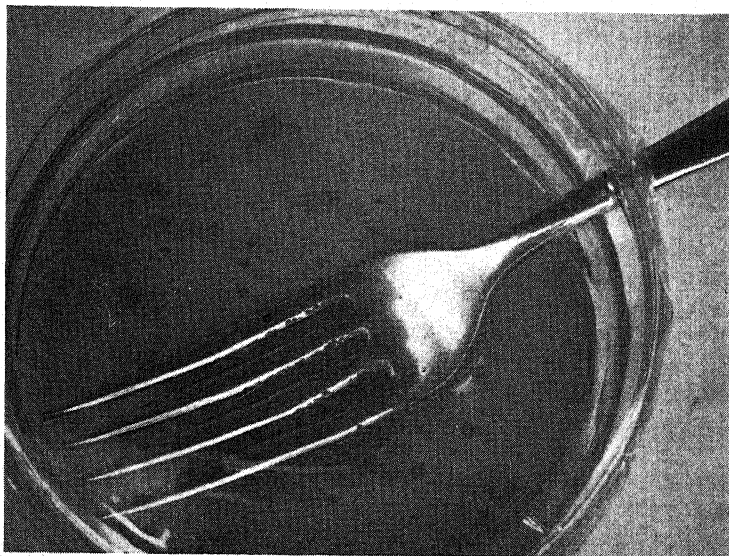


Fig. 25-2. Warm, sterile, fluid agar was poured into the plate. The fork was placed in the agar and moved about to dislodge contaminating material. The dish was then covered, the agar allowed to solidify and the dish incubated. The bacteria from the fork developed into colonies which are clearly visible. Among these hundreds of colonies there are undoubtedly pathogenic bacteria. (Photo courtesy of Dr. Richard R. Barton; from *Appl. Micro.*, 1954, vol. 2.)

metal spoons and forks. Not only is expensive dishwashing equipment, with its noise, sloppiness and heat eliminated, but labor and fuel costs are reduced, breakage costs are trifling, and esthetic and sanitary standards enormously improved. Bacteriological studies of paper used for containers and tableware show that the processes used in their manufacture result in a product with negligible content of microorganisms. Of these none is pathogenic. However, paper dishes have not yet been perfected to the point where the public willingly accepts them in place of china and glass.

(b) **Transmission by Foods.** Foods that are moist and not very acid are likely to be excellent culture media for many pathogenic microorganisms: the typhoid bacillus (*Salmonella typhi*) and dysentery bacilli (*Shigella* species); toxin-producing *Micrococcus* (staphylococcus) species; *Clostridium botulinum* (one cause of food poisoning); the hemolytic streptococci (*Streptococcus pyogenes*) that cause scarlet fever and septic sore throat; the diphtheria bacillus (*Corynebacterium diphtheriae*); and others.

**FOODS AND CARRIERS.** The infection of foods with respiratory or enteric pathogens, represented by the few species mentioned above, is mainly by food handlers who are carriers of the organisms and who sneeze and cough over food and/or handle it with unwashed hands.

Foods may or may not be sterilized by cooking. The center of large masses of food is not always raised to a bactericidal temperature by baking or boiling. Further, if the cooked food is infected by handling *after* it has cooled, and is left standing for hours in a warm kitchen, the persons who eat it might (in some instances) just as well drink a culture in the laboratory. In case of doubt, discard the food or, second best, recook it. Always keep perishable foods (a) covered (avoiding contamination) and (b) refrigerated (avoiding incubation).

**EXTRANEOUS INFECTION.** Some foods are infected from outside sources. A good example is seen in oysters taken from beds polluted by sewage. Always look for the *certification number* on packages of uncooked shell fish. Bakers' cream pies, cream puffs, etc., after incubation in a warm show-case, sometimes contain enterotoxin\* (toxin of enteritis) formed by micrococci. The organisms are usually from a food handler's nose, boil or ulcer.

Home-canned foods sometimes contain the deadly toxin of botulism. This toxin is formed during storage in a warm cellar, by *Clostridium botulinum*. The spores of this anaerobic organism are introduced with soil into the jars or cans. The spores germinate and the bacilli grow if the containers were not properly sterilized after packing (see Chapters 34 and 43). Rats, flies, roaches and other vermin can also infect foods.

**INFECTION OF MILK.** Sterilized milk is often used in the laboratory as a culture medium, and it is a good one. It is thus clear why milk, infected by careless dairymen and incubated for hours in the sun on a loading platform, has been the vector of scores of epidemics of diphtheria, typhoid fever, dysentery, scarlet fever and sore throat ("strep throat").

Milk may be infected not only by dirty hands of dairymen and by insanitary implements and polluted water supplies but, like other foods, also by careless handling in the home, by flies, etc. In addition, it may come from cows in-

\* Do not confuse with endotoxin.

fectured with brucellosis (undulant fever), the streptococci of scarlet fever, the rickettsiae of Q fever, or tuberculosis. Many cases of these last four diseases have originated in infected milk.

*Pasteurization of milk*, and modern methods of sanitary handling of dairy products, control by health departments, and constant vigilance through bacteriological examinations have eliminated nearly all of the dangers from milk. Never drink unpasteurized milk or uncertified raw milk.

(c) **Transmission by Hands.** Milk supplies and the food in any kitchen may become infected from the *hands* of careless milkers, dairymen or cooks who are carriers of infectious microorganisms.

Washing the hands after defecation or urination or blowing the nose is a partial safeguard against transmission of intestinal and respiratory diseases but careless and ignorant persons are often very lax in this respect. Pasteurization of milk and the thorough cooking of foods (followed by prompt eating or prompt refrigeration) are other safeguards. Persons who handle foods for restaurants or institutions, as well as dairy workers, should be required by law to pass bacteriological examinations and are so regulated in many communities. However, enforcement is difficult and expensive. There is danger from enteric and respiratory infections in food handlers, but little from venereal diseases unless in an acute or active stage.

The practice of shaking hands doubtless transmits many pathogenic intestinal and respiratory organisms. If hands are to be *held* they should be *clean*! (Fig. 25-3.)

(d) **Transmission by Droplets of Saliva, Mucus, etc.** The mucous secretions of the nose, throat, mouth and lungs, all combined to some extent with saliva, constitute one of the most formidable vectors of disease. Pneumococci, streptococci, meningococci, diphtheria bacilli and tubercle bacilli, as well as influenza virus, poliomyelitis virus, measles virus, and other organisms of respiratory disease, are thus transmitted. Saliva, and with it oral, nasal and

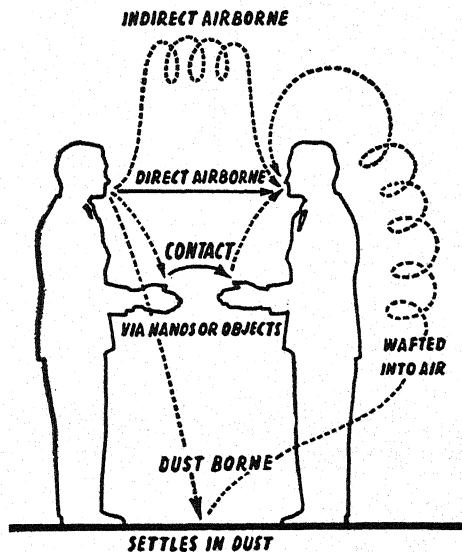


Fig. 25-3. Transmission of infectious microorganisms of oral and respiratory tracts from one person to another. Absent from the picture but active, nevertheless, are contaminated foods, water and milk. (After F. Schwenker, from "Surgical Supervisor," July 1947. Courtesy of the American Sterilizer Co.)

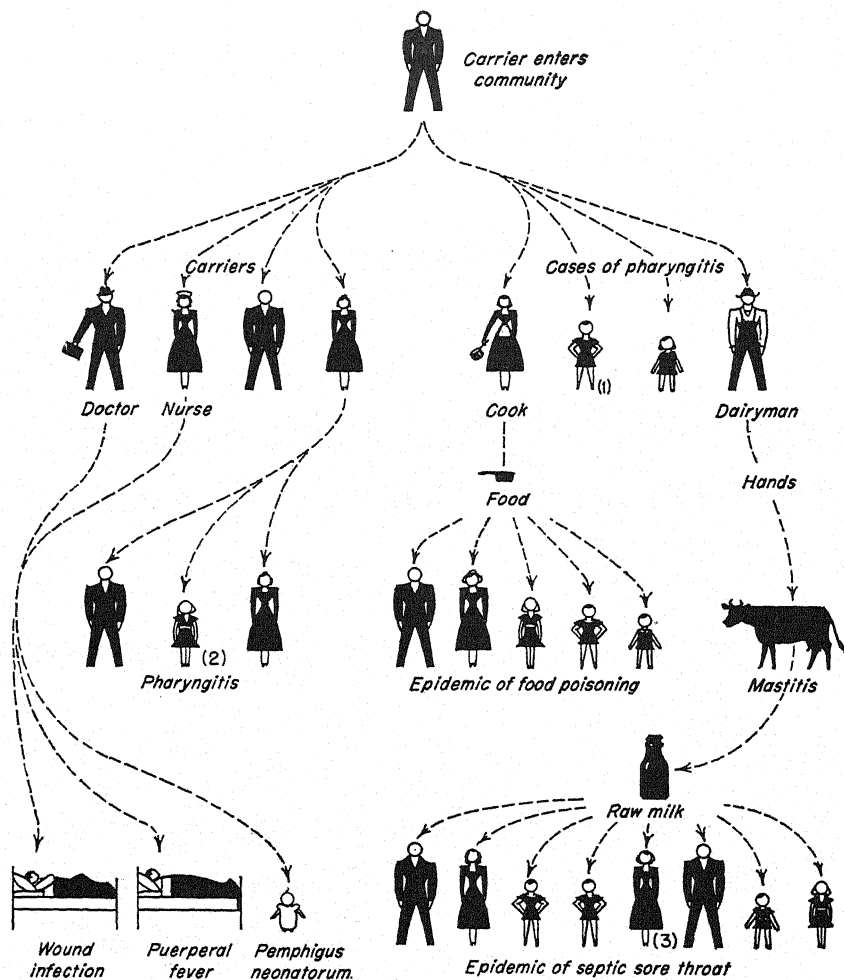


Fig. 25-4. Spread of streptococcal infection. (1) Develops middle ear infection; (2) develops rheumatic fever; (3) develops subacute bacterial endocarditis. (From Witten: Microbiology with Application to Nursing, 2nd ed., McGraw-Hill, Blakiston Division.)

pulmonary mucus, are of the greatest importance in disease transmission (Fig. 25-4).

We are all very careless in our habits in regard to saliva, far more so than we like to realize. The case has been stated vividly by a famous physician: "If infection by contact is of such very great importance in the fecal-borne diseases, how much more important must it be in diseases in which the infective agent is found in the secretions of the nose and mouth, as is the case with diphtheria, scarlet fever, smallpox, mumps, measles, whooping cough, tuberculosis, influenza, and cerebrospinal meningitis. Every one avoids feces and urine, but it is only the very few who have any objection to saliva.

"Not only is the saliva made use of for a great variety of purposes, and

numberless articles are for one reason or another placed in the mouth, but for no reason whatever, and all unconsciously, the fingers are with great frequency raised to the lips or to the nose. Who can doubt that if the salivary glands secreted indigo the fingers would continually be stained a deep blue."

Droplets of saliva are presumably responsible for much disease transmission. Sneezing or coughing in public without a handkerchief is reprehensible but commonplace (Fig. 25-5). Every cough or sneeze results in a microbe-laden spray. The spray droplets remain suspended for some time in the air and may be carried many feet by draughts. They land on food, lips, hands, furniture. The droplets usually become dry.

**DROPLET NUCLEI.** The mucus-coated bacteria which they contain then constitute what are called *droplet nuclei*. These float about through the air like dust particles.

**AIR DISINFECTION.** The possibilities of disinfecting air in public meeting places, operating rooms, etc., have been the subject of intensive and large-scale investigations. The two methods giving most promise are irradiation with ultraviolet light and the use of bactericidal vapors, sometimes called *aerosols*. Both are strongly bactericidal but neither is of significant value for practical purposes except in special situations. These are discussed in more detail in the Chapter on Microorganisms in the Atmosphere.

(e) **Transmission by Dust.** Little imagination is needed to understand how disease may be transmitted by dust. Particles of saliva or sputum containing microorganisms fall to the floor, bedding or clothing, and dry quickly. If not exposed to excessive heat or sunlight or other unfavorable influences, the organisms in the droplet nuclei may survive for considerable periods. When the dust is stirred up, persons inhaling it or getting it into operative or accidental wounds may suffer an attack of disease. Probably the respiratory diseases like tuberculosis, pneumonia, diphtheria and scarlet fever are often transmitted by such means, since the organisms involved resist drying and



Fig. 25-5. Unstifled sneeze explodes a cloud of highly atomized, bacteria-laden droplets. Some droplets travel at such high speed that they are streaks even at  $1/30,000$  of a second. (Courtesy of M. W. Jennison, Depart. of Plant Sciences, Syracuse University.)



exposure to diffuse daylight. Good examples of dust-borne fungal diseases are coccidioidomycosis and histoplasmosis (see Chapter 4).

The dust in places where psittacine birds (parrots, etc.) are raised and sold is a source of much serious and often fatal infection with the virus of "parrot fever" (*psittacosis*), since the virus occurs in feces and nasal secretions of infected birds. These dry and are scattered as dust about the building.

In barracks and hospital wards dust and lint from clothing and bedding are important means of disease transmission, especially of respiratory infections. One method of controlling this is to impregnate bedding with imperceptible oils which tend to keep the dust from flying about. Floors and sweepings are also oiled. The oil merely controls dust. It does not kill microorganisms (see Chapter 41). Bactericidal sweeping compounds (oiled, disinfected sand or sawdust) are now commonly used.

(f) **Transmission by Direct Contact.** This means of disease transmission needs little comment. Obviously, if one rubs against infectious material he runs a risk of infection. Usually it is easy to guard against such an eventuality, especially if one avoids transferring the contagium to its special portal of entry. One does not voluntarily come into physical contact with feces, sputum or the visible sores or pustules of infected persons. If, in the course of professional or home-nursing duties, this cannot be avoided one should wear rubber gloves or wash and disinfect the hands immediately afterward, without touching anything first. However, an innocent kiss may transmit tuberculosis, pneumonia, scarlet fever, diphtheria and other respiratory-borne diseases, and doubtless does, with tragic results especially to infants and young children and very old persons. Common examples of contact-transmitted diseases of adults are syphilis and gonorrhea, both spread by coitus, the former by kissing also.

**Domestic Environments and Diseases.** Infectious diseases are usually much more frequent and widespread in crowded, unsanitary, living quarters than in clean, spacious dwellings. This is well illustrated by insect-borne diseases like typhus (body lice) and plague (rat fleas) which are notoriously associated with low-grade living conditions; often as a result of wars. It is equally true of respiratory diseases and of enteric infections. Microorganisms spread by oral and nasal secretions as in sneezing and coughing and by soiled hands, can much more readily be transmitted from person to person in a close, crowded, cold and damp room, than in a spacious, well-ventilated, warm and dry apartment.

Infection by enteric viruses (polio, hepatitis) and other microorganisms of the intestinal tract (dysentery, typhoid and related bacilli; intestinal worms and protozoa) are obviously transmitted by feces-soiled hands, clothing, soil, water or food. It is very significant that a direct correlation has been shown to exist between many of these diseases and the *availability of ample clean water for domestic purposes*; especially for washing of hands and installation of sanitary plumbing (Fig. 25-6).

## 2. BIOLOGICAL TRANSMISSION OF DISEASE

Biological transmission of infectious agents differs from the foregoing mainly in that the vectors are living animals or arthropods. The outstanding exceptions to this are human blood and blood derivatives.

(a) **Human Blood and Blood Derivatives.** Blood not infrequently contains pathogenic microorganisms. In certain infectious diseases the etiologic agents circulate in the blood stream for varying periods. Typhoid bacilli are readily found in the blood during the first week of the disease. Meningococci not infrequently occur in the blood, even in the absence of meningitis. Rickettsiae are present in the blood during typhus and Rocky Mountain spotted fever and can readily be transmitted by carelessly handled instruments, needles, syringes, etc., also by blood-sucking insects. Many viruses (polio, yellow fever, dengue, encephalitis, and so on) and protozoa (malaria parasites, trypanosomes of sleeping sickness and others) also circulate in the blood.

**HOMOLOGOUS SERUM JAUNDICE.** A very important virus occurring over long periods in the blood of apparently healthy persons is that of *infectious hepatitis* or *homologous serum jaundice*. The virus can be transmitted in blood of donors; in gamma globulin used to prevent diseases like measles; in blood-bank blood; and by syringes, needles, instruments not properly sterilized, and the like. Very rigid precautions must be taken in handling any human blood, tissues or derivatives thereof, or blood-contaminated instruments to avoid transmission of this virus.

**BLOOD-BANK BLOOD.** Human blood may temporarily have many organisms in it immediately after any severe injury or after some tooth extractions or surgery, or even in the absence of any injury at all. Blood drawn at such times for blood-bank purposes may, *if not properly refrigerated*, contain large numbers of bacteria, because the few bacteria which may have been initially present soon multiply to thousands. Even refrigerated blood may support growth of some psychrophilic organisms. Sometimes blood is contaminated by bacteria introduced by the needle from the surface of the skin. Being recognized, these dangers are pretty successfully avoided.

(b) **Bites of Vertebrates.** Any animal (or human) bite will introduce a mixture of the microorganisms present in the saliva and on the teeth. Such

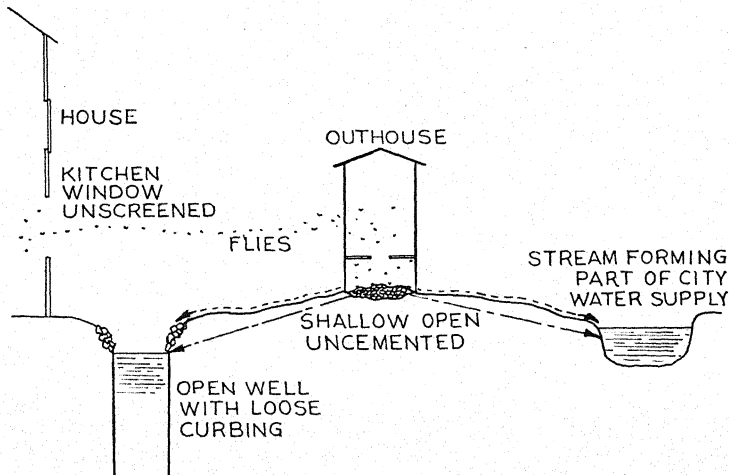


Fig. 25-6. How an unsanitary outhouse may be a source of pollution of a city water supply (over-the-surface washings and underground seepage), a household well (surface washings and underground seepage), and a kitchen (flies).

bites are always infectious and should immediately be opened, cleaned, disinfected and covered with sterile gauze.

The most notorious pathogen transmitted by animal bites is the virus of *rabies* or hydrophobia. Most mammals are susceptible to rabies and can transmit it. Cats, dogs, foxes and wolves are particularly dangerous in this respect. Within the last decade bats: vampire, insectivorous and fruit-eating, have been shown to harbor and transmit rabies among themselves and to cattle, man and other animals.

(c) **Bites of Arthropods.** Many microorganisms are associated with arthropods. There are several diseases, the sole natural means of transmission of which is the bite of arthropods. In 1878 a domestic mosquito (*Culex quinquefasciatus*) was shown to transmit the worm *Filaria bancrofti*, agent of one form of *filariasis* (a notorious symptom of which is *elephantiasis*). The classical observations of Smith and Kilborne in 1893 on transmission of Texas fever of cattle by the cattle tick (*Boophilus annulatus*) were the first on tick transmission of protozoan disease. Certain mosquitoes (*Anopheles*) and other insects were later found to transmit malaria (Fig. 25-7). Usually, but not always, each disease has its own specific insect vector.

Several arthropod-borne pathogenic agents are listed in Table 16.

(d) **Insect Feces.** Cockroaches were shown as early as 1914 to transfer cholera vibrios in their intestines for at least forty-eight hours after feeding on human cholera feces. Ants transmit cholera, and probably other enteric diseases, in the same manner. Flies have long been under indictment for the same crimes.

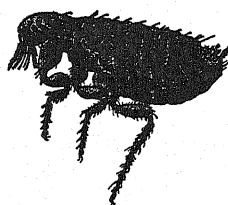
The feces of lice infected with typhus rickettsiae will infect if scratched into the skin. Feces of infected fleas from rats, "prairie dogs" and similar rodents contain plague bacilli and may contaminate small wounds or scratches. Indeed, many bloodsucking insects may pass infective agents in the feces and may also cause infection by being crushed on the skin near, or in, an abrasion or wound. Engorged ticks on dogs are especially dangerous in this respect because they contain a relatively large volume of blood which can transmit the rickettsiae of Rocky Mountain spotted fever. House flies may transmit poliomyelitis by fecal contamination, as the virus has been demonstrated to occur in flies. However, the vector seems a very unimportant one compared to human feces, which is evidently the major vector of polio.

(e) **Bodies of Insects.** Insects which fly or crawl from unsanitary, unscreened and undisinfected privies to hospitals or to dwellings may mechanically transmit intestinal and other disease organisms on their feet and bodies. In areas where flies abound, especially rural or city slum areas, if there is access to infectious sewage or feces, enteric fevers are usually more prevalent during the summer months when flies are numerous. In places where city sewerage systems are not available, fly-borne disease can be avoided to a large extent by the construction of screened and deep-pit or other sanitary types of privies or, better still, by the installation of sanitary plumbing and septic tanks. Plans and specifications for such structures can be obtained from State Health Departments.

**Vertebrate Animals as Vectors of Disease.** Animals constitute an enormous and ever-present *reservoir* of agents infectious for human beings. Classical examples are rabies (found in many common animal species), bubonic plague



**CONENOSE BUGS**  
*transmit*  
Chagas' Disease



**FLEAS**  
*transmit*  
Plague  
Endemic Typhus  
Dog Tapeworm



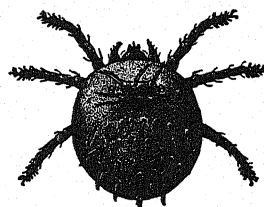
**LICE**  
*transmit*  
Relapsing Fever  
Epidemic Typhus Fever  
Trench Fever  
*cause*: Vagabond's disease



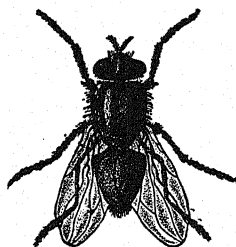
**"SOFT" TICKS**  
*transmit*  
Relapsing Fever  
*cause*: Tick paralysis



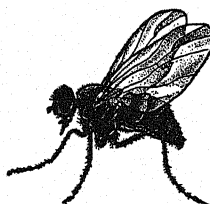
**"HARD" TICKS**  
*transmit*  
Tick-Borne Rickettsioses  
(Rocky Mt. Spotted Fever)  
(Brazilian Spotted Fever)  
(Fievre Boutonneuse)  
(So. African Tick Fever)  
("Q" Fever)  
Tularemia  
Colorado Tick Fever  
Bullis Fever  
*cause*: Tick paralysis



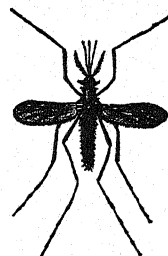
**MITES**  
*transmit*  
Tsumugamushi  
(Scrub Typhus)  
*cause*: Dermatitis



**NON-BITING FLIES**  
*transmit*  
Yaws  
Typhoid Fever  
Dysenteries  
Cholera  
Conjunctivitis  
*cause*: (larvae) Myiasis



**BITING FLIES**  
*transmit*  
Tularemia  
Sand-fly Fever  
Filariases (Loiasis Onchocerciasis)  
African Sleeping Sickness  
Leishmaniasis (Kala-azar, Oriental Sore)  
Bartonellosis



**MOSQUITOES**  
*transmit*  
Malaria  
Yellow Fever  
Dengue  
Filariasis (Elephantiasis)  
Encephalitis

Types of arthropods transmitting human diseases (selected examples).

Fig. 25-7. Types of insects that transmit disease. (From Stitt, Clough, and Branham, "Practical Bacteriology, Hematology, and Parasitology," McGraw-Hill, Blakiston Division.)

**Table 16.** *Some Arthropod-Borne Diseases; Their Vectors and Animal Reservoirs.*

ETIOLOGICAL AGENTS AND DISEASES	PRINCIPAL KNOWN ARTHROPOD VECTORS	COMMON ANIMAL RESERVOIRS
<b>Viruses</b>		
Yellow fever virus	Mosquitoes ( <i>Aedes aegypti</i> , urban; <i>Hemagogus spegazzini</i> and others, jungle)	Man Jungle monkeys
Dengue virus	Mosquitoes ( <i>Aedes aegypti</i> )	Possibly monkeys
Various encephalitis viruses	Mosquitoes ( <i>Culex</i> species); ticks; mites; others	Horses, birds
<b>Rickettsiae</b>		
<i>R. rickettsii</i> (Rocky Mountain spotted fever)	Ticks ( <i>Dermacentor andersoni</i> , <i>D. variabilis</i> .)	Sheep, rabbits, dogs
<i>R. prowazekii</i> (classical typhus)	Lice ( <i>Pediculus corporis</i> )	Man
<i>R. mooseri</i> (endemic typhus)	Fleas ( <i>Xenopsylla cheopis</i> )	Rats
<i>R. orientalis</i> (tsutsugamushi)	Mites ( <i>Trombicula akamushi</i> )	Field mice, etc.
<b>Bacteria</b>		
<i>Pasteurella tularensis</i> (tularemia)	Ticks ( <i>Dermacentor</i> species), Deer-fly ( <i>Chrysops discalis</i> )	Rabbits, various other wild animals
<i>Pasteurella pestis</i> (bubonic plague)	Fleas ( <i>Xenopsylla cheopis</i> )	Rats and prairie dogs
<i>Borrelia recurrentis</i> (relapsing fever)	Ticks ( <i>Ornithodoros moubata</i> and others)	Possibly rodents, other animals
<b>Protozoa</b>		
<i>Plasmodium</i> species (malaria)	Mosquitoes ( <i>Anopheles</i> species)	Man
<i>Typanosoma</i> species (African sleeping sickness)	Tse-tse flies ( <i>Glossina</i> species)	Wild animals

(rats, ground squirrels, etc.), tularemia or "rabbit fever," tsutsugamushi or mite typhus (field mice and other animals) and ornithosis or psittacosis or parrot fever (parrots, "love birds," etc.). Over 80 diseases found in animals are communicable to man. These include viral, rickettsial, bacterial, fungal, and protozoan diseases.

Even the fragile and inscrutable egg is not free from the stigma. Numerous large outbreaks of food infection (diarrhea, etc.) due to *Salmonella* species have been traced to foods made with raw eggs (mayonnaise, etc.). Eggs often contain infectious organisms when laid by an already-infected hen.

Some of the animals closest to man are constant sources of infection. For

example, dogs often harbor hookworms which cause creeping eruption (transmitted by contact with ground soiled with canine feces); *Salmonella* species which cause gastroenteritis (transmitted by feces of various farm animals and poultry); *Leptospira canicola* and *L. icterohemorrhagiae* which cause hemorrhagic jaundice (transmitted by canine and rodent urine); *Rickettsia rickettsii* which cause Rocky Mountain spotted fever (transmitted by ticks: sheep, rabbit and dog); *rabies virus* (transmitted by bites of numerous species of mammals); *Pasteurella tularensis* which cause tularemia (transmitted by flies and ticks which bite rabbits, deer and other wild animals); the fungi of ringworm (transmitted by contact with skin of cattle, horses, dogs and cats); and numerous other pathogenic microorganisms. Two of the commonest serious diseases of animals which affect man are brucellosis and (outside of the United States) bovine tuberculosis. These are transmitted frequently by drinking unpasteurized milk of infected cows. Since brucellosis is also acquired by contact with infected tissues of animals, it is common among veterinarians, and cattle and swine breeders, as well as among employees of slaughter houses.

Rats are well known as vectors of plague, murine typhus, leptospirosis, trichinosis, salmonellosis and other diseases. Rats should be eliminated by poisoning, trapping, and deprivation of food and breeding places through cleanliness and rat-proof construction. The fleas of rats, which transmit plague and murine typhus particularly, may be temporarily exterminated by dusting runways and places of refuge with DDT.

#### REFERENCES

- Anonymous: Wood Ticks. U. S. Dep't. of Agriculture Leaflet No. 387, 1955. U. S. Gov't. Printing Office, Washington 25, D. C.
- Brandly, C. A., and Jungherr, E. L.: Advances in Veterinary Science. Academic Press, New York, 1953.
- Bureau of Entomology and Plant Quarantine: Insects. The Yearbook of Agriculture, 1952. Government Printing Office, Washington 25, D. C.
- Burns, K. F., Farinacci, C. F., Murnane, T. G., and Shelton, D. F.: Insectivorous bats naturally infected with rabies in Southwestern United States. *Am. J. Pub. Health*, 1956, 46:1089.
- Courter, R. D.: Bat rabies. *Public Health Reports*, 1954, 69:9.
- Dack, G. M.: Significance of enteric bacilli in foods. *Am. J. Pub. Health*, 1955, 45:1151.
- Editorial: Control of bacteria in chicken salad. *J.A.M.A.*, 1955, 157:923.
- Eklund, C. M: Ecology of mosquito-borne diseases. *Ann. Rev. Microbiol.*, 1953, 7:339.
- Hagan, W. A., and Bruner, D. A.: The Infectious Diseases of Domestic Animals. 2nd ed. Comstock Publishing Co., Ithaca, 1951.
- Horsfall, W. R.: Mosquitoes—Their Bionomics and Relation to Disease. The Ronald Press Co., New York, 1955.
- Logan, J. A.: Status of insect and rodent control in Public Health. *Am. Soc. of Civil Engineers. Centennial Transactions*, 1953, 634.
- Maxcy, K. F., et al.: Preventive Medicine and Hygiene. 7th ed. Appleton-Century-Crofts, Inc., New York, 1951.
- Ordinance and Code Relative to Eating and Drinking Establishments. U. S. Public Health Service, Publication No. 37, 1950. U. S. Gov't Printing Office, Washington 25, D. C.
- Rosebury, T.: Bacteria Indigenous to Man. In Dubos, R. J., Bacterial and Mycotic Infections of Man. 2nd ed. J. B. Lippincott Co., Philadelphia, 1952.
- Smart, J.: A Handbook for the Identification of Insects of Medical Importance. Ed. 3. British Museum (Natural History), London, 1956.
- Steinhaus, E. A.: Insect Microbiology. Comstock Publ. Co., Ithaca, N. Y., 1946.

- Tanner, F. W., and Tanner, L. P.: Food-borne Infections and Intoxications. Garrard Press, Champaign, Ill., 1954.
- Various Authors: Studies on mites as vectors of encephalitis viruses. *Am. J. Trop. Med. and Hyg.*, 1955, 4:90, 106.
- Watt, J.: Symposium on diarrheal diseases other than amebiasis. *Am. J. Trop. Med. and Hyg.*, 1954, 4:716.
- World Health Organization Technical Report, Series No. 41. International Sanitary Regulations. Geneva, 1952.
- World Health Organization, Technical Report Series No. 47. Expert Committee on Environmental Sanitation. Second Report. Geneva, 1952.

## SECTION 4

### *The Bacteria* (Class *Schizomycetes*)

THIS SECTION describes several groups of important and interesting bacteria. These conveniently illustrate many important biological facts. They have some relation to almost every aspect of human affairs and, in general, constitute a group of organisms knowledge of which is of wide general interest and utility as well as a part of any well rounded education. Here we see exemplified such important biological phenomena as evolution, autotrophy, heterotrophy, photosynthesis, chemosynthesis, anaerobiosis, primitive communal life, adaptations to the widest range of environmental conditions, ecology of microorganisms in relation to each other and higher forms, parasitism, pathogenesis, etc. Many of these phenomena are also seen in other forms of life: microorganisms as well as macroorganisms. It is clear that microorganisms illustrate the basic plan of life.





## The "Slime Bacteria"

### (Order Myxobacteriales)

THE MYXOBACTERIALES (*myxo* is from the Greek meaning slime) are characterized by (1) the formation of a slimy secretion in which large numbers of the bacterial cells live as a sort of community; a so-called "swarm stage"; and (2) alternation between the swarm stage and a "cyst" stage in which the cells aggregate into variously formed groups around which the slime dries, forming a cyst filled with dormant cells. Most myxobacteria are entirely saprophytic but there are some important exceptions. Most species of myxobacteria normally inhabit the sea waters, the soil and decaying organic matter such as old wood, dead leaves, and dung.

1. **The Swarm Stage.** In this stage they multiply by binary fission and secrete a slimy matrix in which they all live together. The cells are usually tapered rods (*fusiform*) reaching a length of from 10 to 15 $\mu$  and a diameter of about 0.5 $\mu$ . They appear to have no distinct cell wall, in this respect resembling many animal cells. The rods differ from ordinary bacilli in being highly flexible, often bending themselves into loops and in this respect resembling the Spirochaetales. The cells move forward also, by means of a slow, gliding motion. Their motion may be due, in part at least, to the secretion of more slime at one end than at the other. There are no flagella.

All the cells in a colony move forward together, secreting slime as they go. "A distinct, firm, hyaline, gelatinous base is secreted by the colony as it extends itself, over which the individuals may move or in which they may become imbedded, and is so coherent a structure that whole colonies may be stripped intact by means of it, from the surface of nutrient agar, for example" (Thaxter). The swarm stage lasts for periods varying from a day or so to a week. Under favorable growth conditions the entire swarm stage growth may cover an area of several square centimeters.

2. **The Encystment Stage.** The rods begin to gather together at different points in the slimy matrix and to heap themselves up. The heaps may become raised above the substratum. In some species this never develops beyond a low, rounded hump. In others it grows into elaborate, branched projections (Fig. 26-1). The rods push to the uppermost portions. The rods then become shortened and rounded and are spoken of as *microcysts*. The gelatinous slime

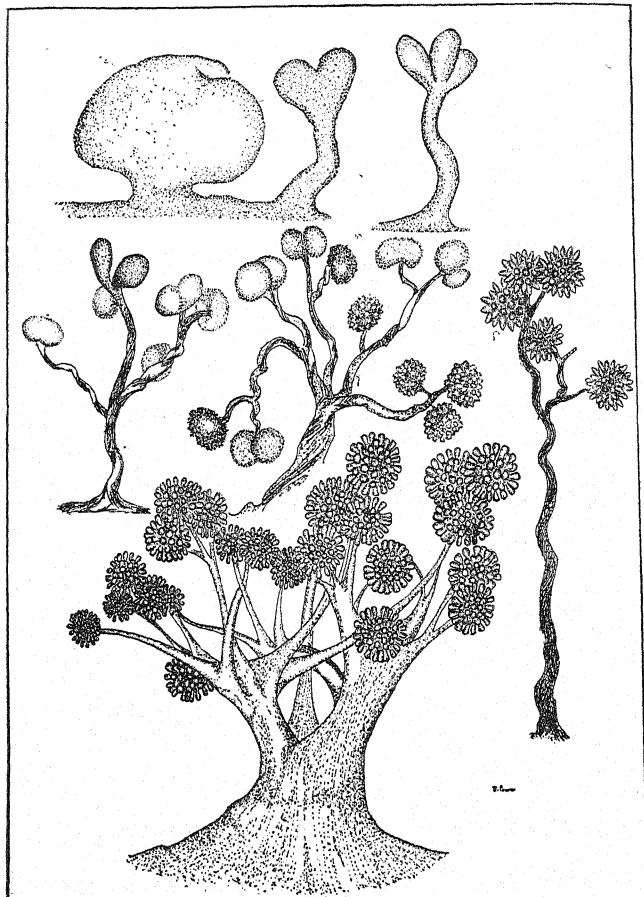


Fig. 26-1. Various mature forms of Myxobacteriales. (Redrawn from Thaxter.)

dries as an envelope about them, enclosing them in a *cyst* or "fruiting body" within which they resist drying and other unfavorable conditions. These fruiting bodies may be as large as 0.1 mm (100  $\mu$ ) in diameter. After a resting stage, the cysts soften and the vegetative rods emerge as new swarm stages, leaving the empty shell.

**Enzymic Activities of Myxobacteriales.** The Myxobacteriales generally produce enzymes capable of hydrolyzing complex molecules. For example, some readily dissolve cellulose, others chitin, still others proteins, etc.

**Genus *Cytophaga*.** Of the various genera of Myxobacteriales, the *Cytophaga* (Family Cytophagaceae) are fairly well known. These organisms inhabit the soil and sea and are of interest because among them are some of the most active *aerobic*, cellulose-decomposing bacteria and because they exhibit several other curious phenomena.

Most *Cytophaga* are strict aerobes, growing well at temperatures between 10 and 30° C; prefer slightly alkaline media (pH 7.5); and are sensitive to acid. An important species pathogenic for commercial fish (*C. columnaris*)

has been cultivated readily on simple media containing peptone (*tryptone*), yeast and meat extracts and sodium acetate. Such media may be fluid or solidified with agar. The colonies of these organisms are beautifully stellate or arborescent, especially when floating in a fluid medium (Fig. 26-2).

Their motility is of the creeping and flexing types and is exhibited only in contact with some fixed or solid surface. Sometimes they swing by one end from a fixed surface and oscillate like a pendulum. It is of interest that a bacteriophage (*myxophage*) active in the lysis of *C. columnaris* has been demonstrated; the first to be observed in any species of Myxobacterales.

**Cultivation of Saprophytic Myxobacterales.** The initial isolation of most of these organisms is not difficult. One method is to place pellets of sterilized rabbit feces close together on the surface of sifted, fresh soil in covered dishes and to keep the whole quite moist for a week or two at about 35° C. Many species grow well at 10° to 20° C. Pure cultures of some species may be obtained on rabbit-dung agar or on infusion agar. The field is an interesting one for the student of cryptogamic botany.

For initial isolation of cellulose-utilizing, soil *Cytophaga* an initial *enrichment* in a solution containing inorganic sources of all necessary elements except carbon will suffice. Such a solution may consist of:

Ingredient	Grams
KNO <sub>3</sub> .....	0.1
K <sub>2</sub> HPO <sub>4</sub> .....	0.1
MgSO <sub>4</sub> .....	0.02
CaCl <sub>2</sub> .....	0.01
FeCl <sub>3</sub> .....	0.002
Water.....	1000

Adjust pH to 7.5.

To this are added some bits of filter paper (cellulose) as a source of carbon.

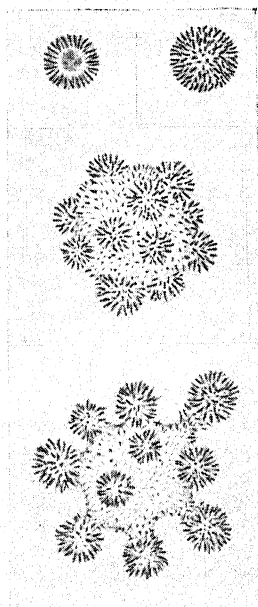


Fig. 26-2. *Cytophaga columnaris*. Clusters (or stars) formed in 0.5 per cent tryptone solution. Living material. Semi-diagrammatic ( $\times 125$ ). (Garnjobst, L., in J. Bact., vol. 49.)

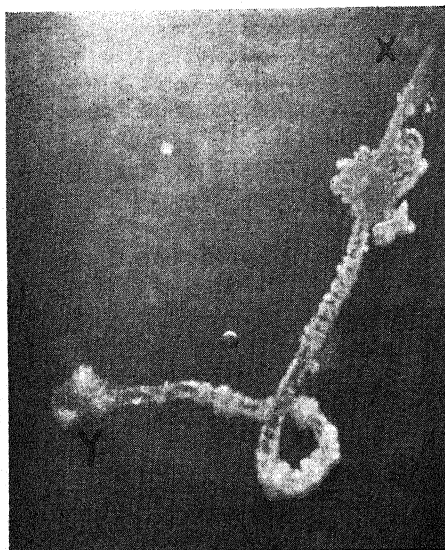


Fig. 26-3. A motile colony of *Bacillus* (sp.). The colony originated at X and, during the course of about 3 days at room temperature, traveled to Y. A portion of the colony then made a small circular journey before the agar was too dry. (About life size.) (Preparation by Dr. Elizabeth I. Parsons. Photo courtesy of Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.)

At least one species of *Cytophaga* (*C. fermentans*) has been described as facultatively anaerobic. It cannot utilize cellulose, agar or chitin but does use simple sugars and starch. It requires complex organic sources of nitrogen, and accessory substances such as the vitamin thiamine.

**Relationship to Other Forms of Life.** We have already indicated certain points of similarity between the Myxobacteriales on the one hand and the Spirochaetales and certain protozoa on the other. These resemblances are illustrated in the flexibility and active bending motions of the rods of myxobacteria in the swarm stage and the apparent absence of differentiated cell wall. The translatory motion of the individual rods of slime bacteria is suggestive of the same type of motion found in sulfur bacteria (*Beggiatoaceae*), in diatoms (*Navicula*), and algae (*Oscillatoria*). The secretion of slime, a striking character of the Myxobacteriales, is also found in the Cyanophyceae, in true bacteria, in the iron bacteria and in the sulfur bacteria. The remarkable communal existence of the Myxobacteriales, in which cells may aggregate for the common aim of transportation, reproduction, and dissemination, is reminiscent of higher plant or animal life, as exemplified in *Volvox* and *Pandorina*, although there is no differentiation of functions among the cells of slime bacteria.

In this connection we may mention the curious communal motility seen in certain species of spore-forming, aerobic rods (genus *Bacillus*). Examples are *B. alvei* and *B. rotans*. These organisms exhibit motility of their colonies similar to that of colonies of slime bacteria. This mobility may express itself as either a rotary motion, or the entire colony may exhibit a migratory mobility, moving over the agar at the rate of 0.01 mm per second (Fig. 26-3). Motility is first noticeable as unorganized, slow motion of masses of cells which finally move forward together. It is of interest that the motion is almost always counterclockwise. How explain this, and how explain the exceptions? One cannot help wondering if this motile colony might not represent a prim-

itive attempt at communal motility which, however, finds greater expression in the higher order of Myxobacteriales.

In the development of a common, slimy colony and tall fruiting stalks with a resistant encysted stage, we find a surprising degree of similarity between Myxobacteriales and those beautiful creatures, the *Mycetozoa* (or Myxomycetes) ("slime animals").

**The Mycetozoa.** In certain groups of these animals a swarm stage is found, which consists of a mass of *living protoplasm* (not inert slime) in which are many *nuclei*. These masses are capable of ameboid motion and, like true amebae, can ingest *solid particles* of food. These are distinctly *animal* characters. The creatures live on rotten logs, etc., in much the same situations as myxobacteria and move about in the moisture and shade like amebae. After several days of such existence they cease to move and reproduction begins. The protoplasm sends up *stalks* on the tips of which *sporangia* (spore-bearing cysts) are formed, in a great variety of the most graceful and delicate forms and of the most brilliant colors. Each sporangium contains many spores and each spore contains a single nucleus and is surrounded by a *cellulose* wall. The spores are dispersed by the wind. In water, each spore germinates, forming a naked, ameba-like creature. These forms multiply rapidly by cell division. They later *coalesce* or fuse, a presumably sexual function, eventually to form the *multinucleate swarm stage*.

The resemblance between these two forms of life is so close, yet so superficial, as to suggest the idea that a comparative study in morphology and function might have been in progress.

#### REFERENCES

- Bachman, B. J.: Studies on *Cytophaga fermentans*, n.sp., a facultatively anaerobic lower Myxobacterium. J. Gen. Microbiol., 1955, 13:541.
- Buchanan, R. E., and Beebe, J. M.: The Myxobacteriales. In Bergey's Manual of Determinative Bacteriology, 6th ed. The Williams & Wilkins Co., Baltimore, Md., 1948, p. 1005.
- Carr, L. G.: Mycetozoa, animal-plant organisms. Sci. Monthly, 1941, 53:175.
- Ordal, E. J., and Rucker, R. R.: Pathogenic myxobacteria. Proc. Soc. Exp. Biol. and Med., 1944, 56:15.
- Sussman, M.: The biology of the cellular slime molds. Ann. Rev. Microbiol., 1956, 10:21.
- Thaxter, R.: On the Myxobacteriaceae, a new order of Schizomycetes. Bot. Gaz., 1892, 17:389; 1904, 37:405.

## The "Sheathed" Bacteria (Order Chlamydobacteriales) and "Stalk-Forming" Bacteria (Suborder Caulobacteriineae)

THE NAME Chlamydobacteriales\* refers to the order of sheath-forming bacteria. It was given because of the presence of a cylindrical sheath or membrane inside of which the organisms live. Chlamydobacteriales are aquatic, saprophytic, filamentous forms. They are important as (1) scavengers in sewage; (2) causes of obstructions in water pipes.

**Structure.** The sheath of *Sphaerotilus*, one of the commonest forms of Chlamydobacteriales, is an excretion product, in some respects like a capsule, but a more distinctly differentiated structure than a capsule. The sheath is soft and flexible, looking and behaving much like a clear, cellophane or paper cylinder such as a drinking tube. Its composition is not fully known. It may be mucilaginous; it is not chitinous.

**Growth.** When the growth of *Sphaerotilus natans* (a species common in sewage) is young, the filaments resemble hyphae of coenocytic molds, though bacterial in dimensions (about 1.5 to 2.0 $\mu$  in diameter). As the growth matures, the protoplast becomes divided up into bacilli from 2.5 to 16 $\mu$  long with lophotrichous flagella. These multiply by binary fission. The resulting motile bacilli slip out at the ends of the sheaths or are liberated at the sides as the sheaths disintegrate (Fig. 27-1). Sometimes the young cells cling to the outside of the sheath of origin and grow off at an angle. This is called *false branching*. In one variety, called *S. dichotomus* (possibly identical with *S. natans*), the false branching appears to be dichotomous.

*Sphaerotilus natans* is aerobic (though requiring very little oxygen), is non-sporeforming and is heterotrophic though it can use inorganic or organic sources of nitrogen. It does not require vitamins.

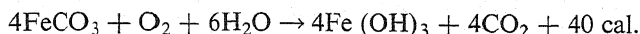
**Sphaerotilus (or Leptothrix) and the Iron Bacteria.** For many years certain filamentous bacteria occurring in iron-bearing, natural waters have been thought to oxidize the iron as a source of energy and to deposit the oxidized

\* *Chlamydo* is from the Greek word meaning a cloak.

iron in their sheath. They have been called "iron bacteria." A widely-known species is called *Leptothrix* (or *Sphaerotilus*) *ochracea*.

The yellow or reddish colored slime found in the beds of iron-bearing waters is nearly always due to ferric hydroxide in the sheaths of these and related iron-accumulating bacteria growing on the stream bottom. *Living*, iron-accumulating bacteria are seldom found in such iron deposits since the incrustation of the membrane with iron interferes with their reproduction.

The role of iron in the physiology of these organisms is interesting but not wholly clear. According to a logical view, only those organisms which oxidize *inorganic, ferrous* iron compounds as a source of energy should be classed as true, iron bacteria. A reaction often given to explain this process is:



A number of similar bacteria utilize *organic* compounds containing iron, but do not oxidize the iron itself. Such organisms, utilizing only the *organic* radical of iron-containing compounds, should not, according to Winogradsky, be classified as true iron bacteria since their energy is obtained by oxidation of the organic matter, *not* the  $\text{Fe}^{++}$ .

Those species oxidizing  $\text{FeCO}_3$  will necessarily deposit *much* waste  $\text{Fe}(\text{OH})_3$  in the sheath since the energy yield is only 10 calories per gm. mol. of  $\text{FeCO}_3$  and only 8 to 10 per cent of this is used by the organisms. Organisms oxidizing only the organic radicals of iron compounds obtain so much energy from the organic matter that relatively small amounts of the ferrous food are needed and *little or no iron* is deposited in the sheath. This organic iron is probably oxidized by free oxygen, extraneously to the cell, and yields no energy to the cell. Such bacteria are not true, "iron bacteria."

**Genus *Crenothrix*.** *Crenothrix polyspora*, a very common and representative species of Chlamydobacteriales, starts life as a single, nearly spherical, non-motile cell about  $2\mu$  in diameter which divides by fission and forms a chain, much as do streptococci. They differ from streptococci in that the cells elongate as they divide, so that the row shortly consists of bacillary forms. They exude a mucilaginous, tubular sheath about  $0.2\mu$  in thickness. The

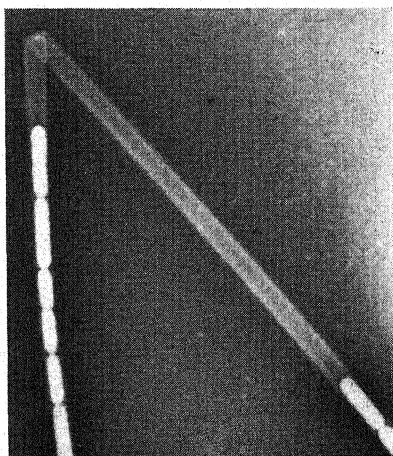


Fig. 27-1. *Sphaerotilus natans*, showing chain of bacillary cells inside their transparent, flexible, tube-like sheath. (Photo courtesy of Dr. J. L. Stokes, Western Utiliz. Res. Br., Agr. Res. Serv., U. S. Dept. of Agriculture, in J. Bact., 1954, vol. 67.)



sheath soon hardens and becomes impregnated with ferric hydroxide. This suggests a true iron bacterium. The topmost cells are continuously being pushed out of the mouth of the sheath by the growing cells below, the filament having attached itself by one end to some solid object.

Very often, in older plants, fission of the uppermost cells takes place in three planes before their liberation from the sheaths. The increase in volume within the sheath forces the latter to expand at the upper end so that a trumpet-like appearance is produced. This is a striking characteristic of *Crenothrix polyspora*.

*Crenothrix* can live inside piped water systems, if the water does not contain too much dissolved oxygen and does contain iron. Like *Sphaerotilus*, *Crenothrix* may or may not be a true iron bacterium but is truly an iron-accumulating bacterium. It forms large gelatinous masses that clog the pipes. When the mature plants die under such conditions, they may decompose, with a resultant bad odor and taste that cause discouraging days in the "complaints" office of the head engineers of water purification plants.

**Systematic Relationships of the "Sheath-Formers."** The relations of these organisms to other groups of organisms are not entirely clear. The order as a whole seems to be more bacterial than mold-like or alga-like. It is not photosynthetic. It is entirely aquatic while most molds are not. In the matter of thread formation the Chlamydobacteriales resemble molds but the threads are much tinier than any mold threads and are of the same order of magnitude as bacteria. The absence of readily demonstrable nuclei is also a distinctly bacterial character. The cells, removed from their sheaths, are distinctly bacterial.

Where branching is present, it is seen to be false branching due to adhesion of filaments to one another and not to an actual branching of individual cells as is seen in molds or Actinomycetales. The resemblance to molds is again seen to be merely a superficial one.

The Chlamydobacteriaceae have often been classed as "higher bacteria." Actually, if we consider the types represented by *Sphaerotilus* and *Crenothrix*, we find them not so very much higher but, as Ellis says, they may be regarded as bacteria which "have taken the first step toward what may be regarded as the communal life."

#### THE CAULOBACTERIINEAE\*

The systematic position of these bacteria is somewhat difficult to determine. As will be seen, iron accumulation is a feature of some, but sheath-formation is absent. The organisms are characterized by forming *stalks*.

The majority of the Caulobacteriineae have been given little attention by most bacteriologists. They are of importance in studies of marine and aquatic problems such as ecology of fish and aquatic vegetation, rotting of ships and piling, etc. Here we will describe only three species: *Gallionella ferruginea*, *Siderocapsa treubii*, and *Caulobacter vibrioides*.

**Gallionella Ferruginea.** This species forms a *stalk* which, as the plant matures, becomes encrusted with  $\text{Fe}(\text{OH})_3$ . It does *not* form a sheath. It is said by some to be a true, iron-oxidizing bacterium but this is doubtful. Its exact taxonomic position is at present obscure.

\* Caulo is from the Latin word *caulis* for stalk.

The cells of this organism are bean-shaped and about  $0.5\mu$  by  $2\mu$  in size. From the concave side of each cell there is excreted a flat, mucilaginous ribbon or stalk. As each cell divides, branching of the stalk occurs so that complex tangles, or rosettes of long stalks, are formed. The stalks are sometimes 0.2 mm to 0.3 mm in length (200–300  $\mu$ ; Fig. 27–2).

The stalks of *Gallionella* have the remarkable habit of twisting so that they resemble a loosely coiled rubber band. Large amounts of iron are later deposited in these stalks, giving them the appearance of a series of loops or string of beads. The twisting habit renders identification of *Gallionella* easy, since no other organism of similar character is known to twist in just this way. As stated by Thimann "—the gallionellas are more notable for their excreta than for themselves."

This organism is found in nature as widely distributed as *Sphaerotilus*. Like other iron-accumulating bacteria, it can multiply in water pipes, and often causes extensive deposits and incrustations of iron which may eventually occlude the pipes.

*Siderocapsa treubii* is a cocco-bacillus about 0.5 by 2  $\mu$ . This organism envelops itself in a thick, slimy or gelatinous, extracellular substance. This does not appear as a tangle of stalks, but in the form of a compact, flat mass attached to some object such as a stone, the underside of a leaf floating in a stagnant pond, or a glass slide immersed to collect growths of such organisms in streams. The gelatinous material may be regarded as a modified stalk or sheath. Sometimes these growths are quite extensive and iron is deposited in



Fig. 27–2. *Gallionella ferruginea*. A shows the curved cells at the ends of twisted, apparently ribbon-like excretion bands. These bands have not yet become heavily encrusted with  $\text{Fe}(\text{OH})_3$ . Microphoto at a magnification of  $\times 1120$ . (From Cholodny, in Starkey, J. Am. Water Works Assoc.) B shows the organisms and the bands (here encrusted with  $\text{Fe}(\text{OH})_3$ , as seen with the electron microscope at a magnification of  $\times 12,000$ . The excretion bands are seen to be fibrillar in structure and to exhibit a segmented pattern. The fibers appear to be fragile and brittle. (Photo courtesy of Drs. A. E. Vatter and R. S. Wolfe, in J. Bact., 1956, vol. 72.)

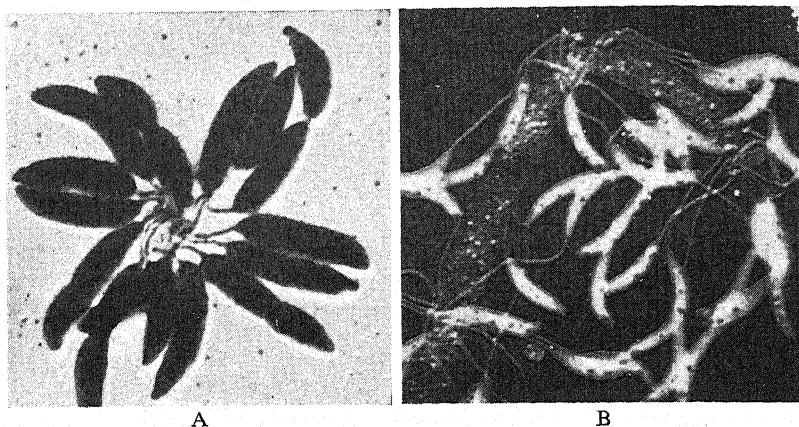


Fig. 27-3. Electronographs of *Caulobacter*. A, *C. vibrioides*, showing stalks attached at a central point, forming a characteristic "rosette." ( $\times 12,500$ .) (Courtesy of Dr. E. A. Grula, in J. Bact., 1954, 68:498.) B, *Caulobacter* sp., showing stalks attached to, and apparently inserted into, cells of *Bacillus megatherium* (a common, saprophytic sporeformer of dust, water, etc.) Note the small size of *Caulobacter* in comparison with *B. megatherium*. ( $\times 6000$ .) (Courtesy of Prof. Dr. A. L. Houwink, Delft; in A. V. Leeuwenhoek J. Micr. and Serol., 1955, vol. 21.)

large amounts around them. They are probably important in the fouling of pipes, but have often been overlooked because their growth is not so extensive as that of filamentous forms. Some question whether *Siderocapsa* is truly a bacterium.

**Caulobacter Vibrioides.** The structure of the stalk of this organism is so distinctly different from both of the foregoing that it should probably be removed to an entirely different group. The body of the bacterium is spindle- or banana-shaped, curved, and pointed at both ends. It often contains granules of volutin, fat and "cell sap." The organisms occur in the general aquatic habitats of Chlamydobacteriales, *Gallionella*, etc., and are of the same order of size and structure as bacteria in general. They multiply by transverse, binary fission, are non-sporeforming and, when young, motile with polar flagella. They have a distinct capsule or slime layer. They may be cultivated in pure cultures using much the same procedures and organic media as are used for other Caulobacteriineae and Chlamydobacteriales.

The peculiarity about the stalk is that it is not an excretion but a distinct *part of the cell itself*; a narrow, flexible, tubular outgrowth closely associated with, or possibly (as the cell matures) *including*, the polar flagellum. This stalk-like outgrowth apparently contains live protoplasm. At the tip is an enlarged, button-like, hold-fast, by which the organism attaches itself to solid objects. In electronographs, fine fibrils are often seen extending from the hold-fast.

These protoplasmic stalks may be very short but are frequently many times as long as the bacterial cells. Often these stalks are attached to, or even (in a possibly parasitic manner) *inserted into* living (later, dead!) cells of certain species of *Bacillus*. This seems to be a unique method of parasitism (Fig. 27-3).

REFERENCES

- Houwink, A. L.: Caulobacter: Its morphogenesis, taxonomy and parasitism. *Antony van Leeuwenhoek J. Micr. and Serol.*, 1955, 21:49.
- Meier, F. E.: Plankton in the Water Supply. *Ann. Rep't., Smithsonian Inst.*, 1939, p. 393.
- Stokes, J. L.: Studies on the filamentous sheathed iron bacterium *Sphaerotilus natans*. *J. Bact.*, 1954, 67:278.
- Strandskov, F. B.: Slime forming organisms. *J. Am. Water Wk. Assoc.*, 1948, 40:1299.
- Thimann, K. V.: *The Life of Bacteria*, Chapter 21. The Macmillan Co., New York, 1955.

## The Sulfur Bacteria

THE TERM "sulfur bacteria" has had various meanings: bacteria which store up globules of elemental sulfur, intracellularly, as reserve food material; bacteria which reduce or oxidize sulfur compounds. As used here it signifies bacteria (a) which *oxidize* sulfur or its inorganic compounds (except sulfates) as a source of energy; and (b) which *reduce* sulfates, utilizing them as an acceptor for elemental hydrogen or hydrogen removed from organic or inorganic substrates. Many of these also store elemental sulfur intracellularly (see Table 17).

Sulfur available to microorganisms exists in various stages of oxidation and reduction in inorganic compounds; ranging from the most reduced,  $H_2S$ , through elemental S, thiosulfates, tetrathionates, etc., to the most oxidized form, sulfates. Any of these, except sulfates, may be oxidized as energy sources by various bacteria; and any, except  $H_2S$ , may be reduced by still other bacteria.

**Groups of Sulfur Bacteria.** The sulfur bacteria may, for convenience of discussion (not as a recognized arrangement), be divided into five groups, four of which comprise oxidizers of sulfur and its compounds (except sulfates). The fifth group contains species which reduce sulfates. For completeness Table 17 includes, as a sixth group, a variety of bacteria which decompose organic sulfur compounds, producing  $H_2S$  (putrefaction). These are not regarded as true sulfur bacteria and are not discussed in this chapter.

### A. SULFUR OXIDIZERS

1. PHOTOSYNTHETIC. Two groups of sulfur oxidizers comprise *Athiorhodaceae*,\* *Chlorobacteriaceae*\* and *Thiorhodaceae*.\* All of these contain bacteriochlorophyll or chlorophyll-like photosynthetic pigments. They may be rod, vibrio or spiral-shaped, much like true bacteria. In the presence of  $H_2S$  the *Thiorhodaceae* store elemental sulfur in the form of conspicuous granules inside the cells. It is later oxidized to  $H_2SO_4$ . (See reactions below.) The *Chlorobacteriaceae* do not store sulfur granules intracellularly but excrete the sulfur in free form outside the cells. The *Athiorhodaceae*, as their name implies, neither store intracellular sulfur granules nor release free sulfur into the surrounding medium. Some oxidize thiosulfates, some oxidize elemental

\* In the suborder Rhodobacteriineae, order Eubacteriales.

hydrogen, but most of them are heterotrophs and use organic sources of energy. These photosynthetic bacteria are more fully discussed in Chapter 29.

2. NONPHOTOSYNTHETIC. (a) SULFUR STORERS. This group includes two other families of organisms which are *sulfur-storers* but *not photosynthetic*. One family (Beggiatoaceae) consists of *filamentous* forms. These filaments float free and unattached and have no sheath. One species (*Thiothrix nivea*) resembles *Crenothrix*, the iron bacterium (see Chapt. 27), in forming a sheath and in attaching itself to solid objects.

The other family (Achromatiaceae) in this group consists of large, ovoid or rod-shaped cells, *not forming filaments*.

(b) NONSULFUR STORERS. This group of sulfur oxidizers, being simple, non-sporeforming, non-branching, non-sheathforming, gram-negative rods, are classed in the genus *Thiobacillus*.\* In this genus sulfur, while used in the

Table 17. *Relations of the Sulfur Bacteria.*

OXIDIZE SULFUR AND ITS INORGANIC COMPOUNDS				REDUCE SULFATES	PRODUCE H <sub>2</sub> S FROM ORGANIC SULFUR COMPOUNDS
INTRACELLULAR SULFUR GRANULES		NO INTRACELLULAR SULFUR GRANULES			
PHOTO- SYNTHETIC	NOT PHOTO- SYNTHETIC	PHOTO- SYNTHETIC	NOT PHOTO- SYNTHETIC		
Thiorhoda- ceae	Beggiatoaceae (filamentous) Achromatia- ceae (non- filamentous)	Chlorobac- teriaceae Athiorho- daceae	<i>Thiobacillus</i>	<i>Desulfo- vibrio</i> <i>Sporovibrio</i>	Various pathogenic and saprophytic (putrefactive) spe- cies: <i>Proteus</i> , <i>Serra- tia</i> , <i>Clostridium</i> , etc.

form of H<sub>2</sub>S as well as in an elemental form as a source of energy, is not held inside the cells. On being released during the oxidation of H<sub>2</sub>S, it accumulates as a free scum or precipitate in the medium. It is afterwards further oxidized to H<sub>2</sub>SO<sub>4</sub>.

#### B. SULFATE REDUCERS

A fifth group of highly specialized sulfur bacteria (sulfate reducers) is characterized by the power to utilize sulfates as hydrogen acceptors in respiration. The sulfates are reduced to H<sub>2</sub>S. This group is comprised chiefly by the genera *Sporovibrio* and *Desulfovibrio*.†

The group of sulfur bacteria, as defined above, is seen to be quite heterogeneous and to recruit membership from widely scattered sources among the Schizomycetales.

**Habitat of Sulfur Bacteria.** Sulfur-utilizing bacteria, photosynthetic and non-photosynthetic, sulfur-storing and non-sulfur-storing, oxidizing and re-

\* In the suborder Eubacteriineae, order Eubacteriales.

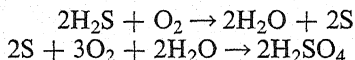
† Suborder Eubacteriineae, order Eubacteriales.

ducing, are found in sewage and other polluted waters, in decomposing organic matter, and in swampy soils all over the world where putrefactive organisms are releasing  $\text{H}_2\text{S}$  from dead plants and animal wastes, or where sulfur-reducing species (*Desulfovibrio*, etc.) are reducing sulfates to  $\text{H}_2\text{S}$ . Some are found around free sulfur deposits. Some occur in acid coal-mine waters; others in garden soil.

The photosynthetic varieties develop best (or only) when exposed to light. In certain sunlit, polluted bays they multiply to such an extent as to cause the entire body of water to look red or purple. Certain autotrophic, photosynthetic species grow in hot sulfur-springs, and others around sulfur mines.

### SULFUR OXIDATION

**Beggiatoaceae and Achromatiaceae.** These non-photosynthetic, sulfur-storing bacteria thrive in sewage and brackish muds. They utilize hydrogen sulfide and sulfur as sources of energy, probably according to the equations



*Beggiatoa alba*, a representative species of these organisms, common in sewage, is autotrophic. *B. alba* requires free oxygen as indicated in the equations above. The acid combines with chlorides, metals, etc., to form sulfates. Sulfates are valuable as the principal sulfur compound available to higher plants.

*Beggiatoa* show creeping and waving movements suggestive of the alga *Oscillatoria*. Typical filaments of *B. alba* range in diameter from 3 to  $50\mu$  and up to several millimeters in length. Reproduction of *B. alba* is by fragmentation of the filaments. Fission also occurs, as in true bacteria. When plenty of  $\text{H}_2\text{S}$  is present the organism stores colloidal globules of sulfur in the cells, giving the organisms a distinctive, milky appearance (Fig. 28-1). When  $\text{H}_2\text{S}$  is scanty the stored S is utilized. When neither  $\text{H}_2\text{S}$  nor stored S is available the organism dies.

**The Genus Thiobacillus.** Thiobacilli are classed with the true bacteria and are typical, autotrophic, chemosynthetic Eubacteriineae. Thiobacilli are small, gram-negative, non-sporeforming, rod-shaped bacteria, some of which are motile, others non-motile. They thrive in mud, sea water, boggy places, coal-mine drainage, and so on, where sulfur and its compounds occur as a result of chemical action or due to protein decomposition or the sulfate-reducing activities of *Desulfovibrio* and related species.

*Thiobacillus* oxidizes sulfur or its inorganic compounds in various ways.

1.  $5\text{Na}_2\text{S}_2\text{O}_3 + \text{H}_2\text{O} + 4\text{O}_2 \rightarrow 5\text{Na}_2\text{SO}_4 + \text{H}_2\text{SO}_4 + 4\text{S}$  (*Thiobacillus thioparus*). Probably this organism forms intermediate compounds like polythionates in the course of this oxidation:

2.  $2\text{Na}_2\text{S}_2\text{O}_3 + \frac{1}{2}\text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Na}_2\text{S}_4\text{O}_6 + 2\text{NaOH}$ .

3.  $2\text{S} + 3\text{O}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{H}_2\text{SO}_4$  (*Th. thiooxidans*).

Among the most interesting and completely studied species of this genus are *Thiobacillus thioparus*, *Th. thiooxidans* and *Th. denitrificans*. These are strict autotrophs. Solutions like the following meet all of their nutritive requirements:

H <sub>2</sub> O.....	100.000 ml
S.....	1.000 gm or
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> .....	0.500 gm
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	0.030 gm
KH <sub>2</sub> PO <sub>4</sub> .....	0.025 gm
CaCl <sub>2</sub> .....	0.050 gm
FeSO <sub>4</sub> .....	0.001 gm

*Thiobacillus thioparus* oxidizes sodium thiosulfate, hydrogen sulfide or sulfur aerobically. The last is transformed quantitatively into sulfuric acid.

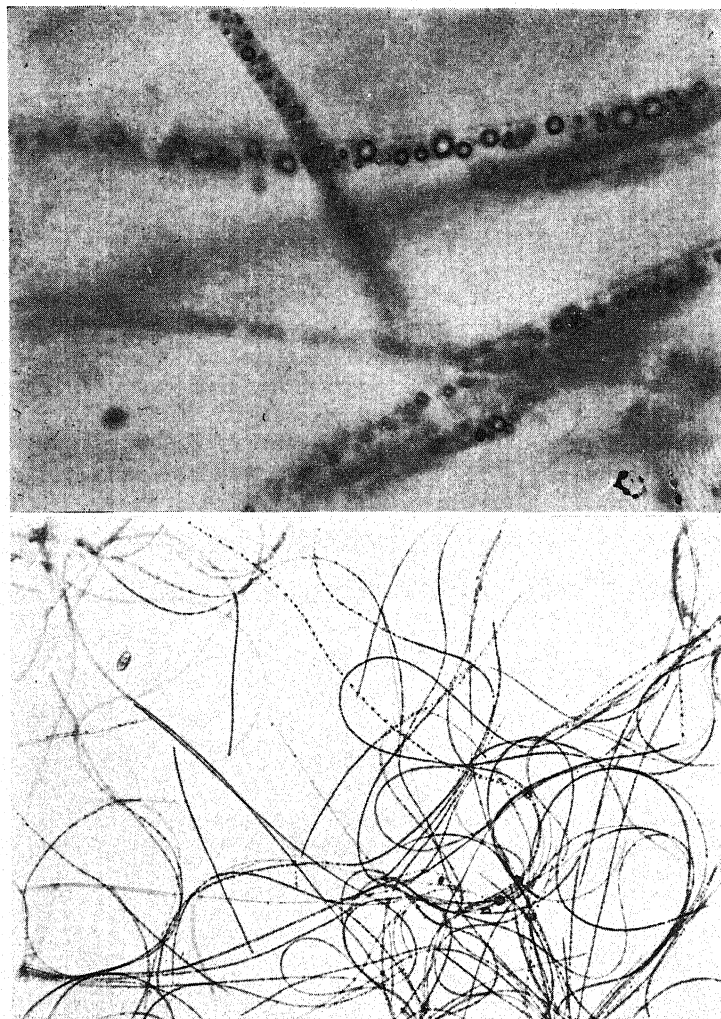
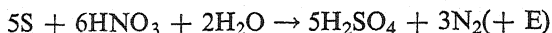


Fig. 28-1. *Upper*, portions of actively motile threads of *Beggiatoa alba* from a picture originally made at a magnification of 2,770. Numerous sulfur vacuoles are distributed throughout the length of the continuous thread. *Lower*, a tangled mass of living *Beggiatoa*, as seen under a 10 X apochromatic objective and 15 X ocular. (Johnson and Baker, J. Cell. and Comp. Physiol., vol. 30.)



*Thiobacillus denitrificans* oxidizes hydrogen sulfide, etc., under strictly anaerobic conditions, using nitrates as hydrogen acceptor and reducing them to nitrites. The metabolism of *Th. denitrificans* is of especial interest since this represents one of the factors responsible for losses of fertility in certain soils: *denitrification*, or reduction of nitrates.

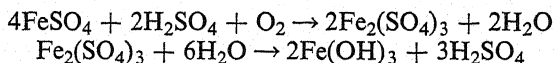


*Thiobacillus thiooxidans* oxidizes sulfur to sulfuric acid aerobically. As sulfuric acid is formed in considerable amounts, it might be thought that the organisms would quickly inhibit their own further growth. This species, however, is of interest in having a great resistance to acid. It is "distinctive in that it is able not only to tolerate but to produce higher concentrations of acid than any other living organism yet known" (Starkey). Some growth is said to occur at a pH of 1, and it grows readily at pH3.

Free sulfur is deposited by most species of *Thiobacillus*, especially *Th. thioparus*, in granules *outside* the cell proper and forms a scum or precipitate in flasks of medium. Most of the species oxidize it further to sulfuric acid.

An interesting physiological question arises as to how sulfur particles, water-insoluble, pass through the bacterial cell wall. No extracellular enzymes are known which fluidify sulfur. A plausible idea is that the sulfur, which must be present in very small (colloidal) particles, is soluble in certain lipid (fat-like) components of the cell wall. Sulfur first dissolves in these and so enters the cell. It is conceivable also that other water-insoluble (or slightly soluble) substances may enter the cell by analogous methods.

*Ferrobacillus ferrooxidans*. This important, strictly autotrophic micro-organism has most of the properties of the genus *Thiobacillus* but does not readily oxidize sulfur compounds. It occurs in the acid (pH 2.3) seepage water of coal mines. Such water contains much ferrous iron. Unlike *Thiobacillus*, this organism gains its energy by the oxidation of ferrous sulfate; a true iron bacterium but not a sheath former. The reactions involved may be similar to the following:

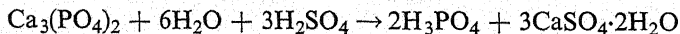


*F. ferrooxidans* can grow in a solution containing:  $(NH_4)_2SO_4$ , KCl,  $MgSO_4 \cdot 7H_2O$ ,  $K_2HPO_4$ ,  $Ca(NO_3)_2$ , and  $FeSO_4 \cdot 7H_2O$ ; pH about 3.5. Note the absence of carbon source.

A closely similar organism, called *Thiobacillus ferrooxidans*, can utilize thiosulfates as well as  $FeSO_4$  as a source of energy. One of these two organisms may be a variant of the other.

#### FUNCTIONS OF SULFUR OXIDIZERS

These sulfuric-acid-forming bacteria are of great importance as agents in the acid-disintegration of various rocks and minerals, with the liberation of such valuable elements as phosphorus, magnesium, and sodium.



Soluble phosphates are among the most important and expensive fertilizers. The transformation of barren alkali soils in desert places to fertile ones may

be brought about through the activities of such organisms under conditions of irrigation.

The sulfuric-acid producers play a very important role in oxidizing offensive sulfur compounds in sewage and other decomposing organic matter.

They are of importance in making sulfur available for other living creatures and thus furthering the *sulfur cycle* (Fig. 28-2).

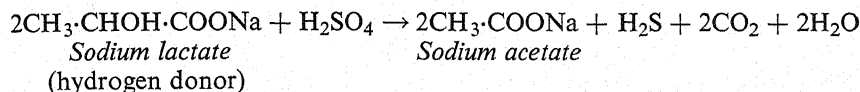
On the other hand, such organisms may prove to be important nuisances because acid production in some situations, as in coal mines and iron water pipes, causes costly damage.

### SULFATE REDUCTION

Reduction of sulfates is the distinctive physiological character of our fourth group of sulfur bacteria. The power of reducing sulfates is not common in bacteria, and with respect to soil fertility, is analogous to nitrate reduction.

**Distribution and Structure.** Sulfate-reducing bacteria are widely distributed, especially in polluted water, the sea and marine muds. They have not been very widely studied but are extremely important. Two of the best known types are curved-rod (vibrio-like) organisms which are classified under the generic names *Sporovibrio* and *Desulfovibrio* (Eubacteriineae). These are simple, unbranched, non-filamentous forms (Fig. 28-3). *Sporovibrio* produces spores. It may be a spore-forming variant of *Desulfovibrio*.

*DESULFOVIBRIO DESULFURICANS*, a well known species which occurs in fresh water, are strict anaerobes and heterotrophic. Organic materials are dehydrogenated and the hydrogen is transferred to sulfates which are reduced to sulfides. An equation illustrating this type of reaction is:



*DESULFOVIBRIO AESTUARII*, found in sea water, can reduce sulfites, thio-sulfate and elemental sulfur to  $\text{H}_2\text{S}$ .

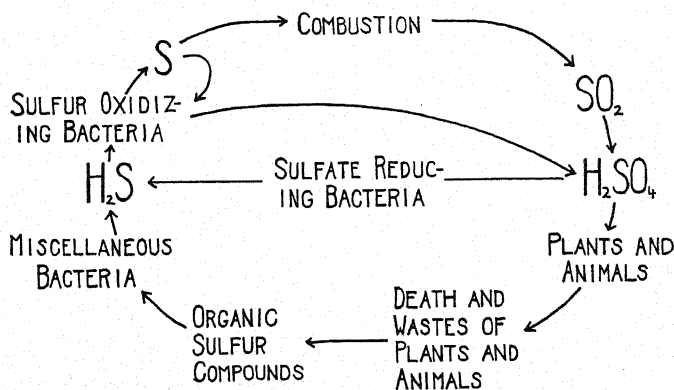


Fig. 28-2. The sulfur cycle. Sulfur in its most reduced form,  $\text{H}_2\text{S}$ , is found in the soil, waters and atmosphere as a result of decomposition of animal and vegetable remains, and volcanic action. Free sulfur occurs as such in some soils, and in mines. Both  $\text{S}$  and  $\text{H}_2\text{S}$  are oxidized by combustion and by microorganisms to sulfates which are used by plants, and these, in turn, by animals. The *reduction of sulfates* by *Desulfovibrio*, etc., is indicated by the right-to-left arrow across the center of the diagram.

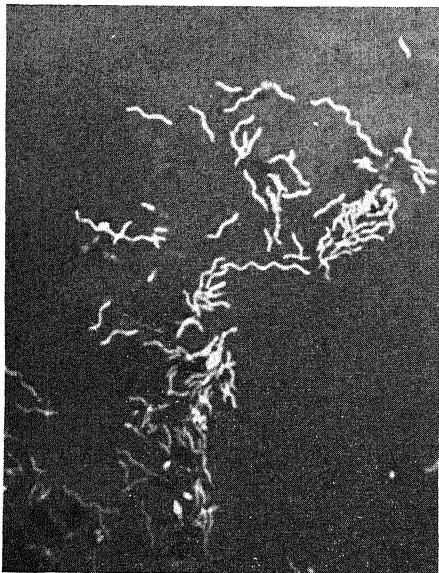
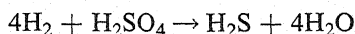


Fig. 28-3. Spiral cells of the sulfate-reducing *Sporovibrio desulfuricans* ( $\times 1660$ ). (From Starkey, J. Amer. Water Works Association.)

Some sulfate reducers can grow autotrophically. They obtain energy from oxidation of hydrogen gas and use sulfate as the hydrogen acceptor. The sulfate is reduced to  $\text{H}_2\text{S}$  by means of the enzyme hydrogenase:



These organisms can also oxidize petroleum hydrocarbons like kerosene, etc., anaerobically, utilizing sulfate as H acceptor. They are of great importance in the petroleum industry because they cause serious losses in the petroleum products.

The low sulfate content of oil-well brines, coupled with the fact that sulfate-reducers may transform certain organic compounds into hydrocarbons, suggests a possible association between these organisms and the formation of petroleum. Further details may be found in the literature cited.

#### REFERENCES

- Fry, B. A., and Peel, J. L., Editors: Autotrophic Microorganisms. Fourth Sympos., Soc. for Gen. Micr., Cambridge Univ. Press, New York, 1954.
- Gleen, H., and Quastel, J. H.: Sulphur metabolism in soil. *Appl. Micr.*, 1953, 1:70.
- Griffiths, M., and Stanier, R. Y.: Some mutational changes in the photosynthetic pigment system of *Rhodospseudomonas spheroides*. *J. Gen. Microbiol.*, 1956, 14:698.
- Harold, R., and Stanier, R. Y.: The genera *Leucothrix* and *Thiothrix*. *Bact. Rev.*, 1955, 19:49.
- Johnson, F. H., and Baker, R. F.: The electron and light microscopy of *Beggiatoa*. *J. Cell. and Comp. Physiol.*, 1947, 30:131.
- Leathen, W. W., Kinsel, N. A., and Braley, S. A., Sr.: *Ferrobacillus ferrooxidans*: a chemosynthetic autotrophic bacterium. *J. Bact.*, 1956, 72:700.
- Sisler, F. D., and ZoBell, C. E.: Hydrogen-utilizing, sulfate-reducing bacteria in marine sediments. *J. Bact.*, 1950, 60:747.
- Starkey, R. L.: Characteristics and cultivation of sulfate-reducing bacteria. *J. Am. Water Wks. Assoc.*, 1948, 40:1291.
- Vishniac, W.: The metabolism of *Thiobacillus thioparus*. *J. Bact.*, 1952, 64:363.
- ZoBell, C. E.: Marine Microbiology. The Chronica Botanica Co., Waltham, Mass., 1946.

## The Photosynthetic Bacteria

THE PHOTOSYNTHETIC bacteria are of two main types: green (Chlorobacteriaceae) and red or purple (Thiorhodaceae\* and Athiorhodaceae). Both types contain photosynthetic pigments. The photosynthetic pigments of these bacteria consist of one or more forms of the bacteriochlorophyll molecule. In some species several carotinoid pigments are also present which give various colors (i.e., red, purple, brown) to the organisms containing them. The carotinoid pigments aid in capturing the energy of visible light in addition to ultraviolet.

✓ **Chlorophylls.** The bacteriochlorophylls belong to the group of chromoproteins. They are remarkably similar to the chlorophylls of green plants. All contain Mg. They are chemically related to the respiratory, red, iron-containing hemoglobin molecule; the blue, copper-containing hemocyanin of lobsters and related animals; and the iron-bearing cytochrome respiratory pigments which are found in all aerobic bacteria.

Green-plant chlorophylls absorb light in wave-length ranges around 6700 Å (blue-green) and also 4570 Å (violet). The green bacteriochlorophyll of Chlorobacteriaceae absorbs waves around 7400 Å (orange-yellow). The bacteriochlorophylls of red and purple species absorb light energy of much longer wave lengths; in the infra-red range, around 8000 to 9000 Å. The brown, red and purple carotinoids, absorbing similar long waves, aid in this absorption of light energy.

**Habitat and General Properties.** The photosynthetic bacteria are found chiefly in marine and aquatic habitats, stagnant lakes, polluted bays, sea water, brackish ditch water, etc. Morphologically they are like typical cocci, bacilli, vibrios and spirilla. They are not filamentous like *Beggiatoa* or *Crenothrix*. They are entirely saprophytic and have no special industrial, agricultural or medical relationships. They are, however, of much interest to the biologist and the biochemist shrewd enough to see their possibilities.

**Use of Photosynthesis.** For example, in these days of scientific search for energy sources mankind investigates solar furnaces (vast, lens-like mirrors to act as enormous "burning glasses"), atomic piles, nuclear reactors and still other mechanisms. Not the least intensive is the effort to determine how chlorophyll collects solar energy and synthesizes, quietly and efficiently,

\* *Thio* is from a Greek word for sulfur; *rhod* is from a Greek word meaning rose-colored.



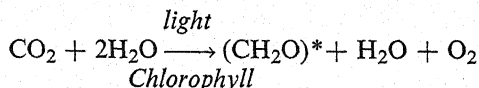
enormous quantities of valuable organic materials in plants (wood, sugar, starch, drugs, vitamins, etc.) mainly from  $\text{CO}_2$  and  $\text{H}_2\text{O}$ .

The structure of the chlorophyll molecule is largely known; it has been isolated and inspected at  $60,000\times$  magnifications; its *location* and *arrangement* in the cell have been determined; its *raw materials* are obvious; its first *end product* has been isolated; what it *does* is clear. But just *how* it (presumably) splits hydrogen from water and transfers it to  $\text{CO}_2$  (though it does so all day long under our very noses) is like the elusive pea in the "3-shell Monte" game: look as closely as we like, "we can't quite figger it out!" The photosynthetic bacteria furnish excellent study material and are much used for such investigations.

An interesting development in our knowledge of photosynthesis is the discovery of some of the first compounds formed by the photosynthetic process. Supplying  $\text{CO}_2$  made with radioactive carbon ( $\text{C}^{14}\text{O}_2$ ) to photosynthesizing algae (*Scenedesmus*), the compounds formed during the first few seconds of photosynthesis were: phosphoglyceric acid (87%), phosphopyruvic acid (10%) and malic acid (3%). A few minutes later in the process, the  $\text{C}^{14}$  was found in many other compounds: amino acids like alanine; sugars like sucrose, fructose and glucose; organic phosphorus compounds; organic acids like succinic acid. The actual synthetic reactions involved are not clear, but the whole synthetic mill, the entire "production line," goes forward smoothly, with lightning-like speed. The first compounds mentioned were formed in five seconds, and many more were formed in workable quantities within fifteen seconds to a minute or more; all in silence, with no visible motion, confusion, odors, slag piles, strikes, wars or social upheavals!

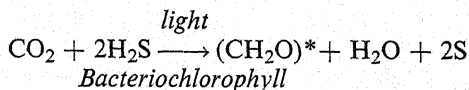
#### PHOTOSYNTHESIS AS A TYPE OF REACTION

1. **Green Plants.** In photosynthesis by green plants hydrogen is removed from water under the influence of sunlight. A simplified form of this reaction is as follows:



Actually, what is being dealt with is electron transfer by which energy is liberated. Any substance which, in the chlorophyll system, will yield hydrogen (an electron), will serve the purpose. In green plants apparently only water will serve.

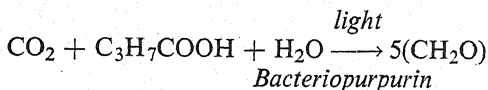
2. **Chlorobacteriaceae and Thiorhodaceae.** The Chlorobacteriaceae and Thiorhodaceae utilize  $\text{H}_2\text{S}$  in photosynthesis. Hydrogen is transferred from  $\text{H}_2\text{S}$  to  $\text{CO}_2$ :



This reaction is exactly analogous to that for photosynthesis by green plants,  $\text{H}_2\text{S}$  substituting for  $\text{H}_2\text{O}$ ; free S being released instead of free  $\text{O}_2$ .

\* This is a hypothetical organic compound, the true nature of which is uncertain.

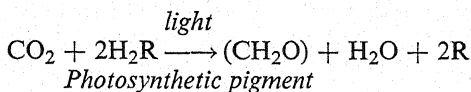
**Athiorhodaceae.** The non-sulfur oxidizing Athiorhodaceae\* use organic sources of H, instead of H<sub>2</sub>S. Thus:



Such organisms do not release sulfur for obvious reasons. Hence the name of the group.\* Some of these bacteria can use molecular hydrogen directly.

**General Type Reaction.** In all cases the hydrogen is incorporated with carbon dioxide in some sort of organic compound generally represented in the reaction formulas as CH<sub>2</sub>O. The process probably varies in the various organisms.

Bacterial photosynthesis is thus seen to be a special case of the type of reaction developed by van Niel for photosynthesis in general:



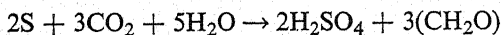
where R indicates the remainder of the molecule from which the hydrogen is abstracted.

Some growth occurs in the dark, but photosynthesis does not occur. In the dark, hydrogen is not transferred to carbon dioxide but to oxygen. This necessitates *aerobic* conditions. Bacterial photosynthesis thus occurs *only under anaerobic conditions* and in the presence of light. The metabolism, in other words, becomes like that of non-photosynthetic aerobic bacteria.

Thus, the photosynthetic feature of the photosynthetic microorganisms seems merely to be added to an ordinary aerobic, oxidative system of metabolism. This suggests that, in the course of evolution, these photosynthetic species were derived from more primitive forms of bacteria with chemosynthetic systems.

As van Niel states: "If, under certain conditions, one of the non-sulfur-storing photosynthetic bacteria† should fail to produce its prominent pigment system, it would thereby become indistinguishable from a typical *Pseudomonas*, *Vibrio* or *Spirillum* species." It is interesting to note that non-photosynthetic mutants of photosynthetic bacteria have been discovered.

**Sulfur Utilization by Bacteria.** The sulfur liberated from H<sub>2</sub>S is stored in the cells of Thiorhodaceae, later to be oxidized to H<sub>2</sub>SO<sub>4</sub>. The free sulfur stored in the cell as sulfur granules is eventually oxidized to sulfuric acid and used in photosynthesis.



The Chlorobacteriaceae release free sulfur into the surrounding medium. This sulfur is likewise used as an electron donor in the assimilation of CO<sub>2</sub>. Some species can utilize polythionates (H<sub>2</sub>S<sub>2</sub>O<sub>6</sub>, H<sub>2</sub>S<sub>4</sub>O<sub>6</sub>).

**Other Relations of Light to Photosynthetic Bacteria.** Some very interesting observations have been made on the dynamic effects of light on certain photo-

\* *Athio* is from Greek and Latin words, meaning without sulfur.

† Athiorhodaceae and Chlorobacteriaceae.

synthetic bacteria. If a beam of light be thrown across a fluid culture of certain species of these organisms, they will quickly leave the darker parts and crowd into the lighter portion. This attraction toward light is called *phototaxis*.

If a spectrum be thrown on a culture of certain motile Rhodobacteriineae the organisms will congregate most thickly in the infra-red portions. The infra-red is the region in which these bacteria *absorb energy* for photosynthesis to the greatest extent. It is of interest that the infra-red and red rays were probably the first to pierce the murky atmosphere of the primitive earth. These bacteria may represent descendants of the first organisms able to make use of such light in a photosynthetic process.

*Thiospirillum jenense* (see family Thiiorhodaceae) has a single, polar flagellum which acts like the propeller of a ship in moving the cell forward. By arranging a patch of shadow so as to move over a swimming cell of *T. jenense* it has been shown that there is a definite point where sensitivity to light is localized. If the shadow moves over the organism from "ahead" a "shock movement" or reversal and change of direction occurs as soon as the *point of insertion* (center of motivation) of the flagellum is covered. The tendency is to stay in the light where energy is available, and to stay in that *color* of light in which, for that organism, energy is most absorbed. No "shock movement" occurs until the sensitive spot near the base of the flagellum is shaded. This localization of sensitivity to light recalls vividly the red "eye spot" of *Euglena viridis* and stimulates speculations concerning the evolutionary origin of eyes in animals.

#### REFERENCES

- Calvin, M., Benson, A. A., and Basham, J. A.: Artificial photosynthesis. *Sci. News Letter*, 1954, 66:387.
- Clayton, R. K., and Delbrück, M.: Purple bacteria. *Sci. Am.*, 1951, 185:68.
- Fry, B. A., and Peel, J. L., Editors: Autotrophic Microorganisms. Fourth Symposium, Soc. for Gen. Micr., Cambridge Univ. Press, New York, 1954.
- Gest, H.: Metabolic patterns in photosynthetic bacteria. *Bact. Rev.*, 1951, 15:183.
- Hill, R., and Whittingham, C. P.: Photosynthesis. Monograph on Biochemical Subjects. John Wiley and Sons, New York, 1955.
- Rabinowitz, E. I.: Progress in photosynthesis. *Sci. Am.*, 1953, 189:80.
- van Niel, C. B.: The culture, general physiology, morphology and classification of the non-sulfur purple and brown bacteria. *Bact. Rev.*, 1944, 8:1.
- van Niel, C. B.: The comparative biochemistry of photosynthesis. *Am. Sci.*, 1949, 37:371.
- van Niel, C. B.: The chemoautotrophic and photosynthetic bacteria. *Ann. Rev. Microbiol.*, 1954, 8:105.
- Various Authors: Carbon Dioxide Fixation and Photosynthesis. Symposia, Soc. for Exp. Biol., Academic Press, New York, 1951.

## The Mold-like Bacteria (Order Actinomycetales)

OF THE VARIOUS orders into which the class Schizomycetes is divided, the order Actinomycetales is one of the most distinctive. It differs from all others in that true, coenocytic branching is normally found in all of its species (Fig. 30-1). None is motile. None forms true endospores though some species form mold-like conidia. The whole order is often referred to as *actinomycetes*.

### THREE FAMILIES OF ACTINOMYCETALES

For convenience of discussion these branching bacteria may be grouped in three families, as shown in the following paragraphs.

**The Family Mycobacteriaceae** is the smallest, containing only the bacterium-like genus *Mycobacterium*. This includes the tubercle bacilli (*Mycobacterium tuberculosis*) and related species. Here, branching is at a minimum. Any short filaments which are formed tend to break up at once into bacillus-like fragments.

These organisms are distinguished by a peculiar staining property called *acid-fastness*. This has been described in Chapter 9. Occasionally acid-fast cells, or fragments, are found in other actinomycetes, especially *Nocardia*, but it is not like the definite, strong and constant acid-fastness of *Mycobacterium*.

**The Family Actinomycetaceae** includes true mycelium-formers. The anaerobic genus *Actinomyces* of this family includes the pathogenic species, *Actinomyces bovis*, causing "lumpy jaw" or *actinomycosis* of cattle, and a few other species which may or may not be pathogenic. In the genus *Actinomyces* the filaments tend to fragment into bacillus-like segments but to a lesser extent than in *Mycobacterium*.

A second genus, *Nocardia*, comprises *aerobic* filament-formers which ordinarily remain unfragmented unless mechanically disturbed. They tend to grow on (and in) the surface of solid media such as agar, rarely producing much aerial mycelium. None of the Mycobacteriaceae or Actinomycetaceae produces spores or conidia.

**The Family Streptomycetaceae** consists of three genera: (1) *Streptomyces*, which form long, aerial mycelia. These are mold-like, coenocytic, non-frag-



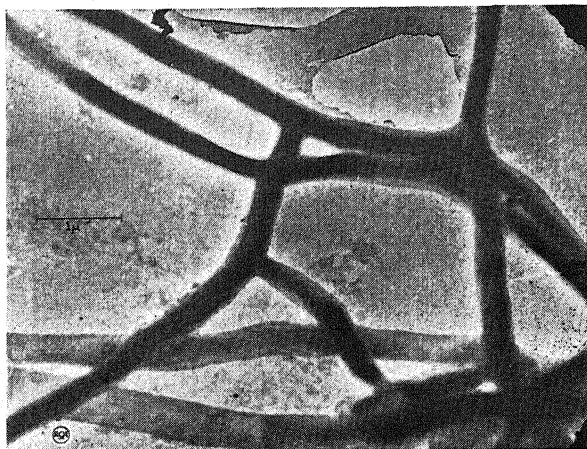


Fig. 30-1. Electronograph of a species of actinomycete showing branching filaments. The irregular shadows in the filaments are of unknown significance. (Courtesy of Dr. Katharine Polevitsky and Dr. R. F. Baker; from unpublished work.)

menting filaments. Conidia are produced in long chains at the ends of the filaments. (2) *Micromonospora*, which are like *Streptomyces*, but produce only a single conidium at the tip of each short, fertile hypha. They produce much less aerial mycelium, growing more on the surface and in the substance of solid media. (3) *Thermoactinomyces* is a small group of species much like *Micromonospora* but, as the name implies, they grow only at temperatures between 50° C and 65° C. The relationships of these groups are shown below.

#### THE ORDER ACTINOMYCETALES (TRUE BRANCHING)

Family Mycobacteriaceae (acid-fast)	} No Conidia
Genus <i>Mycobacterium</i> (fragment; bacterium-like)	
Family Actinomycetaceae	
Genus <i>Actinomyces</i> (fragment; anaerobic)	
Genus <i>Nocardia</i> (fragment; aerobic)	} Conidia
Family Streptomycetaceae (filament formers; do not fragment)	
Genus <i>Streptomyces</i> (well developed mycelia; curled chains of conidia)	
Genus <i>Micromonospora</i> (single conidia)	
Genus <i>Thermoactinomyces</i> (50°-65° C)	

**CONIDIA.** The conidia of the family Streptomycetaceae (often improperly called spores) are not as heat-resistant as bacterial spores, being killed by 10 to 30-minute exposures to 65° C, a temperature only slightly higher than that required to kill the vegetative mycelium. In many species of *Streptomyces* the formation of conidia is accompanied by a corkscrew twisting of the filament so that a very curious, curled appearance is given to the mycelium (Fig. 30-2). The direction and form of the coils are constant for any given species.

**Molds and Actinomycetes.** Striking and constant differences between the mold-like Actinomycetales and the true molds (Eumycetes) are: (1) the minuteness of the filaments of Actinomycetales (1 to 2  $\mu$  in diameter and sel-

dom more than a few millimeters in length; true molds range around  $50\ \mu$  diameter and inches in extent); and (2) the absence of readily demonstrable nuclei in Actinomycetales; both distinctly bacterial characters. Granules of volutin and other substances, as well as vacuoles, are often seen within the older mycelia of Actinomycetales, and have probably been mistaken by some investigators for nuclei.

The colonies of the filamentous Actinomycetales are usually tough, dense-

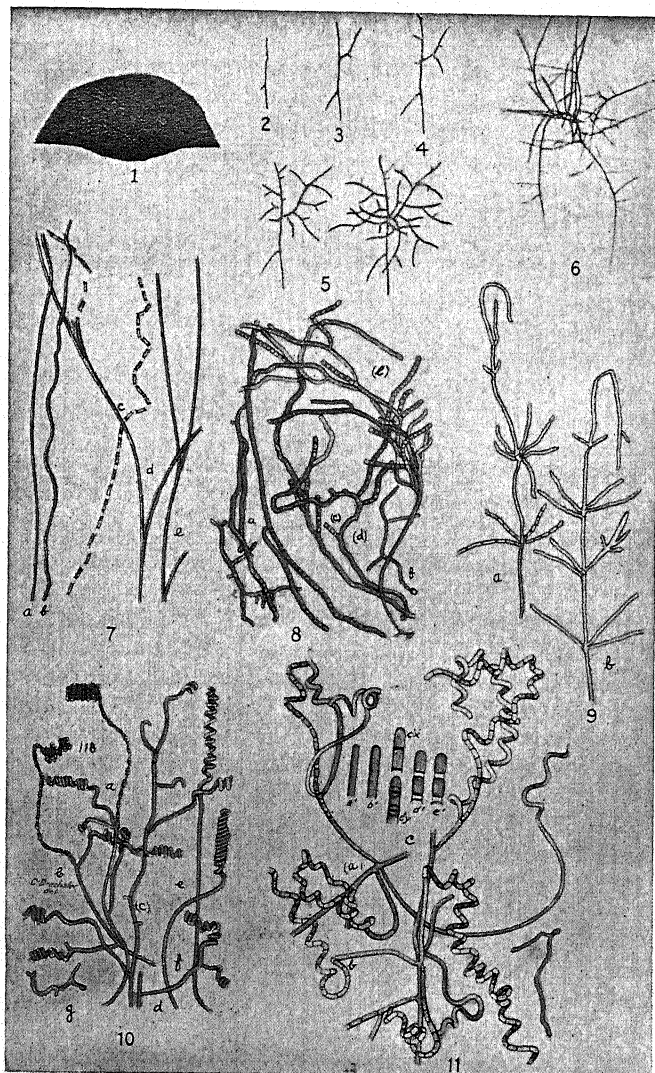


Fig. 30-2. Details of growth and structure of various Actinomycetales. 1, cross section of colony; 2-6, successive stages in growth from a single conidium; 7-11, various types of mature mycelium, showing different kinds of branching, conidia formation, twisting, fragmentation, etc. (1-6 from Lieske; 7-11 from Drechsler and Waksman.)

textured, and often very adherent to the medium. They have a wooly or velvety appearance, due to the mycelial structure. The growth of many species (except *Actinomyces*) is often brilliantly colored: red, orange, yellow. Colonies range in diameter from less than 1 mm to several mm; definitely smaller than the huge colonies of true molds.

There is no apparent differentiation of sexes, and no evidence of sexual phenomena such as are seen in molds.

#### FAMILY ACTINOMYCETACEAE

Most of the members of this family are saprophytes, living in the soil as scavengers, attacking and decomposing complex organic substances of a great variety, such as cellulose, protein, starches, fats, and even carbolic acid, (phenol), naphthalene, rubber and cresol as sources of energy and carbon. Except for these curious sources of carbon and energy their food requirements are simple minerals.

**Genus *Nocardia*.** Of this genus, several species are parasitic in man or animals, causing tuberculosis-like diseases or ulcerative lesions (*nocardiosis*). They are generally aerobic. Most species are soil saprophytes and are frequently found as contaminants in laboratory cultures. The mycelial fragments are often bacillary-looking. There are usually numerous misshapen, clubbed and knobbed forms containing volutin granules.

The colonies of these organisms on agar are usually like those of true bacteria, ranging in diameter from 1 to 10  $\mu$  and being pasty in consistency as a rule. Filaments are inconspicuous or absent, and the organisms in smears often much resemble true bacilli except that branching is sometimes well marked.

**Genus *Actinomyces*.** These differ markedly from *Nocardia* in: (1) being anaerobic or microaerophilic (the only anaerobic organisms in the order Actinomycetales); (2) requiring complex organic foods, such as chopped meat in broth. They are associated with the disease actinomycosis in man and animals. They are much like *Nocardia* in morphology and colony form.

*Actinomyces bovis* occurs as a normal inhabitant in the oral cavity of cattle and other animals and man. Thence it may be introduced into the flesh of the jaws, tongue, etc. by thorns, splinters and the like. Swellings are produced by the growing *Actinomyces* and the surrounding tissues become hard and indurated; hence the colloquial term "wooden tongue" for actinomycosis of the tongue in cattle. Eventually the infected tissue becomes riddled with abscess-like cavities which are filled with pus.

*Actinomyces bovis* tend to form, in the pus, large (0.5 to 5 mm diameter), yellow masses of growth ("sulfur granules") with a central core of matted mycelia, the tips of which project at the exterior much like the spines of a sea urchin or a chestnut burr. These tips characteristically enlarge and become club-shaped. Crushed between two slides, the mycelial and radial structure of the granules is evident. (See Fig. 30-3.)

**"Ray-fungi."** The name of this order is derived from the term *Actinomyces*, first used by Harz in 1878 to describe the organism causing "lumpy jaw" of cattle. *Actinomyces* is derived from Latin words meaning "ray fungus." The name is descriptive of the radial or sunray-like arrangement of the threads of *Actinomyces* which make up the yellow granules ("sulfur granules")

or colonies of the organism found in pus from lesions of actinomycosis (see Fig. 30-3). This radial arrangement is not a common character of Actinomycetales, branching cells being the prime differential character of the order. However, *A. bovis* was the first species to be described and is now the type species of the genus *Actinomyces*.

#### FAMILY STREPTOMYCETACEAE

This family includes the most mold-like of all of the Schizomycetes. These organisms are mainly saprophytes and are active in decomposition of all types of organic material. A few are pathogens in animals or plants. *Streptomyces scabies*, for example, produces a troublesome disease (scab) of potatoes. Some produce valuable antibiotics; e.g., *S. griseus*, streptomycin. Cultivation from soil is not difficult, but slow development may permit overgrowth by other, more rapidly multiplying, bacteria if the latter are present in large numbers in the culture.

The flat, tough, strongly adherent colonies of *Streptomyces* on infusion or extract agar are often papillate, and frequently the surface is thrown into radial folds or ridges. They give off a distinctive, musty odor characteristic of damp cellars and newly turned soil.

Growth is usually best at temperatures around 25° C, although some thermophilic soil species (genus *Thermoactinomyces*) grow well at temperatures as high as 65° C. Unlike most true molds, optimal growth occurs at pH 8 or 9, and is greatly depressed by reactions of around pH 5.

It is obvious that liming of acid soils will encourage growth of the saprophytic actinomycetes, resulting in increased fertility due to the activities of these organisms in decomposing complex organic materials so that other bacteria and farm crops can make use of them. The various species causing "scab" of potatoes and other root crops are also encouraged by this procedure so

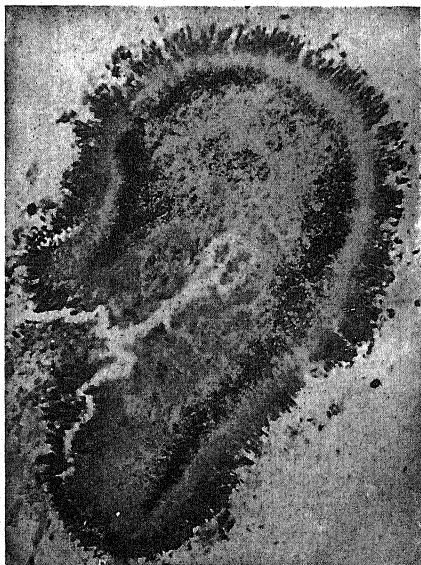


Fig. 30-3. "Sulfur granule" from a case of actinomycosis ( $\times 80$ ). (Wright.) (Photograph by L. S. Brown.)

that one must consider the nature of the crop to be raised and soil pH before indiscriminate liming. As the Streptomycetaceae and other actinomycetes are aerobic, it is apparent why draining swamp lands increases their fertility.

**Streptomyces and Antibiotics.** The genus *Streptomyces* constitutes one of the most extensively investigated groups of bacteria because various species produce antibiotics of value in human or veterinary medicine, in the control of crop diseases and industrial spoilage by bacteria, and in scientific research.

For antibiotic production *Streptomyces* are generally cultivated in deep tanks of aerated fluid media, much as is *Penicillium notatum* for penicillin production. Growth occurs in a flocculent form and antibiotic production is usually good (Chapter 44).

**ACTINOPHAGE.** The growth may undergo lysis. Normally this is *autolysis*, which occurs usually at about the time antibiotic production is best. Destructive, troublesome lysis is sometimes caused by *actinophage* (like bacteriophage) which destroys the growing organisms.

**SELECTION OF PRODUCTIVE STRAINS.** In antibiotics production great care is used to select and maintain strains of *Streptomyces* which produce maximum amounts of antibiotic; to detect and eliminate 'phage, and to find new antibiotic-producing strains or species. Here is a field offering opportunities to all, from the tyro to the advanced expert.

#### FAMILY MYCOBACTERIACEAE

The family consists of only the genus *Mycobacterium*. These are gram-positive, non-motile, aerobic, non-sporeforming rods. The Ziehl-Neelsen stain is of special value in the identification of mycobacteria, since they are unique in being strongly acid-fast. *Morphologically*, they are much like ordinary bacteria except for being somewhat curved and spindle-shaped, and showing beaded or granular cells. The granules are often called Much granules after the first writer to describe them. They have been regarded as forms in a life cycle of tubercle bacillus or nuclear apparatus. The most acceptable view at present is that they are artifacts resulting from staining processes. The mycobacteria branch only slightly, so that X, Y, L and similar forms are sometimes seen. Filament formation is minimal and transitory. Most of them are harmless saprophytes living in the soil and decaying organic matter. None of them forms conidia or aerial hyphae.

**Non-pathogenic Species of *Mycobacterium*.** Saprophytic mycobacteria may be found in properly stained smears of material from preputial secretions (*Mycobacterium smegmatis*) or from folds of the skin as in the buttocks or axillae. Saprophytic acid-fast bacilli are also found in butter (*Myco. butyricum*), in manure (*Myco. stercoris*), or on hay (*Myco. phlei*). (While many species are named, few are fully described and most are synonymous with *M. phlei*). Many species are also found in the soil where they act as scavengers and bring about decomposition of a wide variety of complex organic compounds: proteins, fats, carbohydrates, petroleum, paraffin, etc. Except for the carbon source, their nutrient requirements are relatively simple.

These saprophytic mycobacteria are readily cultivated at 25° to 30° C on infusion agar containing glycerin, or on coagulated egg or serum. Like *Streptomyces*, they will also grow well in simple mineral solutions containing a little asparagine and glucose; i.e., they are close to the autotrophs. Yellowish

or reddish pigments are common. Their growth is fairly rapid as a rule (48 to 96 hours). The colonies of the saprophytic species are usually soft and moist, (pasty or butyrous-butter-like).

Some species of mycobacteria, very much like these saprophytes, are found associated with lesions in the lung resembling tuberculosis. Their etiological relationship to such lesions is not clear. They are often accompanied by tubercle bacilli.

**Mycobacterium Tuberculosis.** This organism, causing tuberculosis, is typical of the genus *Mycobacterium*. It is the most strongly acid-fast species of the genus.

Tubercle bacilli do not form spores, but may remain alive for long periods outside the human or animal body. In dried sputum in dark corners they may live six to eight months. In particles of dried and powdered sputum which can float through the air as dust, they can remain alive for days and may be inhaled. Exposure to sunlight for a few hours kills them and so does pasteurization. This is of importance since they can infect cows' udders and hence the milk, thus transmitting the disease from cattle to persons who drink unpasteurized or improperly supervised (un-Certified) raw milk.

**TYPES OF TUBERCLE BACILLI.** There are several kinds of tubercle bacilli, varying according to the animal infected. For example, there is a human type (*Myco. tuberculosis*, var. *hominis*) and a bovine type (*Myco. tuberculosis*, var. *bovis*) which grow well but slowly (2-6 weeks) on solid media such as coagulated egg, serum or blood, or on glycerin agar at 37° C. A third mammalian type, called the vole\* bacillus, was discovered in 1937. This organism is highly virulent for voles but in man produces a mild immunizing infection. There is also a bird or avian type (*Myco. avium*) which grows well at about 40° C, and turtle (*Myco. chelonae*), fish (*Myco. marinum*) and other cold-blooded animal types which grow well at lower temperatures (18° to 30° C). All look alike microscopically and may be cultivated on similar media. The cold-blooded animal types do not as a rule infect the warm-blooded animals or birds and vice versa. The avian type occasionally infects mammalian species. Human and bovine types seem to infect man and guinea pigs with equal ease but differ in their effect on rabbits, the bovine killing the rabbit within four to six weeks, the human usually not killing at all, or much more slowly. This is of diagnostic value.

#### TUBERCULOSIS

**Tubercles and Tuberculosis.** Tuberculosis is much more common than is generally supposed. It kills a great many more people in these United States each year, even in this enlightened age, than diphtheria, scarlet fever, typhoid fever, mumps, measles, whooping cough and poliomyelitis altogether.

When tubercle bacilli gain a foothold in a susceptible animal or person, the tissues where the bacilli localize immediately begin to react against the organisms in a very characteristic way. Numbers of tissue cells begin to grow around the bacilli in an attempt to incarcerate them or wall them in. A tiny, pearly, gray mass of cells is thus formed, with tubercle bacilli at the center. It is called a *tubercle*. If the resistance of the host is low, the tissues are unable to arrest

\* A vole is a kind of field mouse.

the bacilli, which continue to grow, killing the surrounding cells and destroying the fibrous walls. Numbers of adjacent tubercles may thus coalesce. The dead tissue at the center of such masses of tubercles becomes cheesy and yellowish and is said to be *caseated*. It may rapidly involve the major part of the lung or other infected organ. If the tubercle is in a lung, the necrosis (death of tissue) may extend till it invades and breaks through the wall of a bronchus. The caseous material, which may contain millions of living tubercle bacilli, is discharged with the sputum by coughing (Fig. 9-4). A large cavity is left behind (*cavitation*).

Sometimes the caseous process breaks through the wall of an artery and then hemorrhage of the lung occurs, which may be fatal. In the vast majority of human beings these tuberculous processes tend to heal and to form life-long scars, whether the patient recovers or not.

✓ **Tuberculin Reaction.** Allergic sensitivity to the protein of the tubercle bacilli may be demonstrated in a large proportion of *adults* by introducing *into* (not *under*) the skin the tuberculo-protein in the form of dead tubercle bacilli, or the sterile filtrate of broth in which they have grown, or purified protein derivatives (PPD) extracted from the bacilli. These tuberculo-proteins are called *tuberculin*. At the site of their introduction into the skin a red, itching spot appears in twenty-four to forty-eight hours. The reaction is called a *tuberculin reaction*, and is representative of the delayed type of allergy described in Chapter 23.

**TUBERCULIN REACTION AND RESISTANCE.** When a tuberculous infection progresses to the time when hypersensitiveness appears, a certain degree of resistance to later infection develops. Tuberculin tests become positive at the same time. Evidently the hypersensitive tissues are able to react locally to bind and arrest the further progress of new infection. Similar tests for allergy (using appropriate, specific antigens) are used in diagnosing certain other slowly-progressive infections: brucellosis or undulant fever, tularemia or "rabbit fever," trichinosis, histoplasmosis and coccidioidomycosis (Chapt. 4).

**Immunization Against Tuberculosis.** Many attempts have been made to produce an effective vaccine to prevent tuberculosis. The most widely used and favorably reported immunizing agent is BCG, a variant of a bovine strain of tubercle bacilli of low virulence (Chapt. 22).

**Laboratory Diagnosis of Human Tuberculosis.** Tubercle bacilli often occur in large numbers in the sputum of persons with active pulmonary tuberculosis. For diagnostic purposes the organisms may be demonstrated there or in other material such as urine, pleurisy fluid or feces, depending on the organs affected. The methods available for demonstrating the tubercle bacilli in pathological material are (a) microscopic examination of smears of the material, stained by the acid-fast method; (b) cultivation on media specially adapted to the growth of the tubercle bacillus and (c) by the inoculation of guinea pigs followed by observation of the development of tuberculosis in these animals. These procedures are relatively simple and are described in any diagnostic manual or textbook of medical bacteriology. The essential steps are:

1. Preparation and examination of a smear stained by the Ziehl-Neelsen method.
2. Treatment of the specimen (usually sputum or gastric washings) with an



equal quantity of 5 per cent NaOH to destroy ("digest") the mucin and most of the contaminating bacteria.

3. Neutralization of the "digested" material.

4. Inoculation of the digested material onto media containing egg, milk, blood or serum, with some agent to inhibit contaminants: dyes, antibiotics.

5. Test of virulence by injection of the digested, neutralized material into a guinea pig.

**Mycobacteria and Wetting Agents.** Mycobacteria have simple nutritive requirements and will grow well in mineral solutions containing only 1 or 2 amino acids. A difficulty with such aqueous media is that mycobacteria contain much wax. Therefore they are not easily *wetted*. Consequently aqueous nutrient solutions do not come into intimate contact with the cell surface. In such media the bacilli grow reluctantly in clumps and float like granules of wax on the surface. As a result, nutrition is retarded and growth of tubercle bacilli slow, requiring 2 to 3 weeks and the use of copious inoculum.

The addition of a wetting agent (surface-tension reducent) (for example, one commercially named "Tween-80"\*) permits the preparation of rapidly (2-4 days) growing subsurface cultures in simple aqueous media. These cultures remain in a *diffuse state* manifested by an even turbidity with no *granule* or *pellicle formation*. Growth is easily initiated by *minimal inocula*.

The nutrient solution comes into intimate contact with the outside of each cell and permits rapid diffusion of foods and wastes, with consequent rapid growth.

This principle, of adding wetting agents to cultures of microorganisms, is an important one and is worthy of further investigation in connection with other organisms to facilitate fast, smooth, evenly distributed growth. This is of importance in many bacteriological procedures: agglutination tests, enumeration, etc. Sometimes wetting agents like "Tween 80" contain toxic impurities which are offset by adding a little serum or serum derivative, such as Bovine albumin fraction V (Armour & Co.).

## LEPROSY

This disease is of great interest from several standpoints: (a) historically; (b) because of the mystery surrounding its mode of transmission; and (c) doubt concerning its true etiology.

**Etiology.** In 1874 Hansen described an acid-fast bacillus, morphologically indistinguishable from *M. tuberculosis*, in lesions of lepers. It is called *Mycobacterium leprae*. It is always present in leprosy tissue, and never occurs in normal tissue. For many years this has been the only one of Koch's postulates to be demonstrated in connection with the etiology of leprosy. Demonstration of this organism in histological sections or biopsy material (material cut out during life) is the most useful and conclusive diagnostic procedure.

Several reports of successful inoculation of human beings with leprosy material have appeared. It still is not certain that the *only* infectious agent transferred in these occurrences was the bacillus of Hansen. Moreover, all

\* Tween-80 (Atlas Powder Co., Wilmington, Del.) is a polyoxyethylene derivative of sorbitol mono-oleate. (Prepare 10 per cent aqueous stock solution in distilled water. Do not use after 1 month. Keep in refrigerator.)



of these people were in *constant association* with lepers. Inoculation of leprous tissue into chimpanzees, etc., has never produced an authentic case of leprosy.

An organism which will cause leprosy has never been cultivated, although many claim to have done so. There are several strains of so-called *M. leprae* which may be obtained on ordinary culture media. They have all the properties of saprophytic species of *Mycobacterium*.

**The method of transmission** of leprosy is obscure. Lesions and discharges contain the bacilli in large numbers. Prolonged, close contact appears to be necessary. Persons under 25 are more likely to contract the disease. The incubation period appears to range from a few months to as long as twenty years. In certain regions of the world leprosy is *endemic*, that is, always present. Among these areas are tropical and subtropical Asia and Africa, Polynesia and South America. It is also present in certain areas of Europe (U.S.-S.R., Baltic States, Spain).

There are about 500 known cases of leprosy in the United States, mostly in the National Leprosarium at Carville, Louisiana, and possibly 1000 unknown cases. In this country leprosy appears to *spread* only in particular areas where conditions are favorable. The reason for this is not clear. Clinically, leprosy progresses very slowly, lasting for years in a patient.

**History.** Leprosy is well known to students of the Bible and medieval history. The fear and horror of lepers has been a human tradition since antiquity. In ancient times lepers were excluded from all public contacts and left to die of exposure and starvation by many peoples. They still are excluded and shunned, but provisions for their comfort and well-being are usually adequate. In former days many disfiguring diseases were confused with leprosy: various fungal infections, yaws, protozoal infections, and so on. One of Conan Doyle's most intriguing Sherlock Holmes episodes depicts the tragic segregation of a noble young man because of a suspicion of leprosy. Holmes, with the aid of Watson, dispels the ugly cloud by his usual astute, deductive reasoning, and the victim is found to have nothing but a harmless skin condition called ichthyosis. The horror of leprosy arises from the disfigurement of the body and destruction of tissue, with scar formation, which accompanies it.

The disease is most distinctive in its so-called *lepromatous* phase. Nodules and gross deformities and ulcerations occur in the skin, with thickening, discolorations and wrinkling. Nerves are often affected so that wounds go unnoticed, and revolting and terrifying (to the ignorant and superstitious) disfigurements occur.

#### REFERENCES

- Bloch, H.: Acid-fast bacteria. *Ann. Rev. Microbiol.*, 1953, 7:19.  
Cummings, M. C.: Chapter on Tuberculosis. *Diagnostic Procedures and Reagents*. Am. Pub. Health Association, 1790 Broadway, New York, 3rd ed., 1950.  
Dubos, R. J.: Second thoughts on the germ theory. *Sci. Am.*, 1955, 192:31.  
Editorial: Whereat tuberculosis? *Am. J. Pub. Health*, 1956, 46:895.  
Gross, M.: *Mycobacterium Tuberculosis: Bacteriology, Biochemistry, Laboratory Diagnosis and Chemotherapy*. Published by the Author, Jersey City, N. J., 1955.  
Long, E. R.: The germ of tuberculosis. *Sci. Am.*, 1955, 192:102.  
Rosenthal, S. R.: Standardization and efficiency of BCG vaccination against tuberculosis. *J.A.M.A.*, 1955, 157:801.

- Sams, C. F.: Experiences in immunization against tuberculosis with BCG vaccine in Japan. *Am. J. Pub. Health*, 1954, 44:903.
- Thomson, D.: Tuberculosis—The changing emphasis. *Monthly Bul., Ministry of Health and the Pub. Health Lab'y Serv.*, (London), 1956, 15:99.
- Various Authors. Symposium on B. C. G. *Bull. W. H. O.*, 1950, 2:347.
- Waksman, S. A.: *The Actinomycetes*. *Ann. Crypt. et Phyt. The Chronica Botanica Co.*, Waltham, Mass., 1950.
- Waksman, S. A., and Lechevalier, M. A.: *Guide to the Classification and Identification of the Actinomycetes and Their Antibiotics*. *Williams & Wilkins Co.*, Baltimore, 1953.

## The Spiral, Flexible Bacteria (Order Spirochaetales)

THE TERM spirochete is often used in a general sense to include all members of the order Spirochaetales. It will be so used here. This inclusive and non-specific word must be differentiated from the name of one genus of spirochete (*Spirochaeta*) and one genus of true bacteria (*Spirillum*).

**General Characters and Structure of the Spirochaetales.** The order Spirochaetales is divided into two families: (a) Spirochaetaceae, which includes three genera of relatively large, saprophytic spirochetes: *Spirochaeta*, *Saprosira* and *Cristispira*; (b) Treponemataceae, comprising three genera of relatively small, very slender organisms, some of which are highly pathogenic: *Treponema*, *Borrelia* and *Leptospira*. The nutrient requirements of spirochetes are complex. Only a few species, notably *Leptospira* and *Spirochaeta*, have been cultivated to a significant extent on artificial media.

The spirochetes resemble most true bacteria (sub-order *Eubacteriineae*) in being: unicellular; non-filamentous; non-branching; and without sheaths, sulfur granules, slimy masses like the slime bacteria, or incrustations of any metal. They do not form stalks; are without photosynthetic pigments; are microscopic in size; without readily demonstrable nuclei; and are without definite sex phenomena. They multiply by transverse, binary fission. Some species may multiply by other methods involving filterable granules. None forms pigments, spores, conidia or capsules. They differ from all other bacteria in being both: (a) cylindrical and spiral in form (Fig. 31-1), and (b) flexible and contractile.\*

MOTILITY in most spirochetes is in part due to their helicoidal form and their rotation around the long axis, as well as to slow, snake-like, bending movements. The electron microscope appears to have demonstrated structures that look like flagella on certain species.

FIBRILLAR STRUCTURE. Studies with the electron microscope indicate that the structure of spirochetes is more complex than that of true bacteria. The cells of several species (perhaps all) appear to have large numbers of flagellum-like fibrils which form bundles wound spirally from end to end of the cell,

\* Note the differentiation from genus *Spirillum*, order Eubacteriales which, while spiral, are rigid, and from the Order Myxobacteriales which, while flexible, are not spiral.

in a manner suggestive of the fibers of a twisted Manila hempen rope. These bundles of fibrils appear, in some species, to be inside the cell wall. In some species their regular convolutions, from end to end, produce what looks like a septate or chambered structure, a character formerly assigned to some spirochetes. In one species (*Cristispira*) the fibrils appear to be on the outside of the cell. There they appear to be aggregated and modified into a flat, keel-like membrane called a *crista*.

In some species (*Leptospira*, *Treponema*) the cell appears to be wound spirally around a central, rod-like structure, possibly a bundle of fused fibrils. The arrangement is suggestive of a thin-walled, rubber tube wound spirally around a flexible, springy, plastic wire (Fig. 31-2).

It appears that the fibrils in all species are contractile. This could explain the flexing and wriggling motility of spirochetes. The fibrils (and perhaps other forms of bacterial fibrils) may be thought of, provisionally, as modified, rudimentary or evolved forms of flagella.

**SIZE.** The spirochetes, while in general not much thicker than true bacteria, are usually much longer. In some species of the genus *Spirochaeta* individuals as long as 500  $\mu$  have been described.

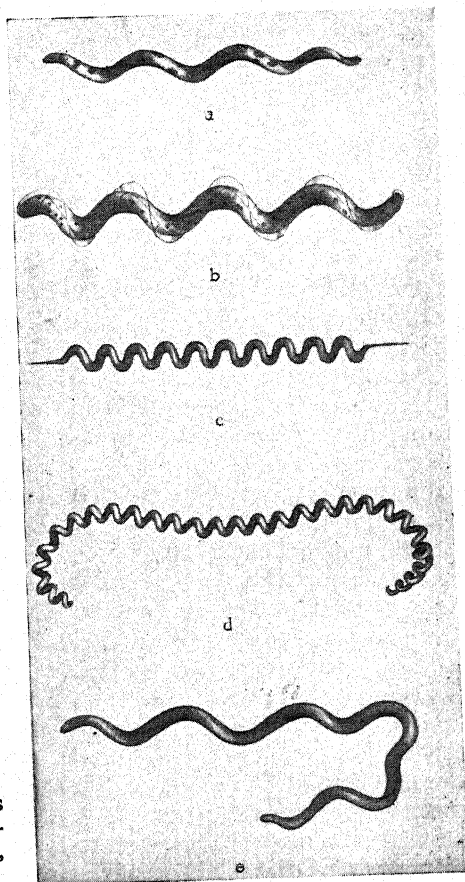


Fig. 31-1. Diagram of various types of spirochete. a. *Spirochaeta*; b. *Cristispira*; c. *Treponema*; d. *Leptospira*; e. *Borrelia*.

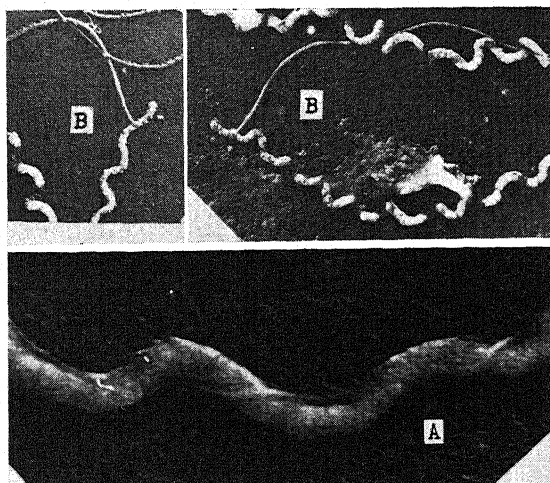


Fig. 31-2. Electronographs of leptospira showing, at *A*, the cytoplasmic cylinder wound helically around a thinner, rigid axistyle; at *B*, the released axistyle which is attached at the ends of the cytoplasmic cylinder. It is suggested that the axistyle is not only the "backbone" of the leptospira but also its means of locomotion, which is probably due to contractility of the axistyle. (Photos courtesy of Dr. J. W. Czekalowski, Univ. of Leeds; from J. Path. and Bact., 1955, vol. 69.)

**MICROSCOPIC STUDY.** Spirochetes as a rule are not readily stained by ordinary dyes like methylene blue, although certain species are exceptions. Some are gram-negative. Giemsa's stain, which colors protozoa, also stains the spirochetes. Improved stains have also been developed. The larger species of spirochete are conveniently observed in unstained preparations, and the method of negative staining sometimes gives satisfactory preparations. The darkfield apparatus is most convenient for observation of the pathogenic species when fluid suspensions are available.

By all microscopic methods granules which may well be associated with reproduction have been seen. The nature of these spirochetal granules is not entirely clear. Some species, especially saprophytic ones, also contain many volutin and fat granules.

**FILTRABILITY AND REPRODUCTIVE GRANULES OF SPIROCHETES.** Certain of the spirochetes (e.g., *Leptospira* and *Borrelia*) are readily filtrable through porcelain or Seitz-type filters which hold back ordinary bacteria. This, of course, distinguishes them from true bacteria. It is thought by some authorities that the filtrability of these organisms is due to the formation, through disintegration or otherwise, of minute granules. Possibly these are the supposed reproductive granules referred to in the preceding paragraph. Material (e.g., lake water) which has been filtered and found, on *microscopic* examination, to contain no visible spirochetes, has later been shown to contain spirochetes by cultural methods. However, microscopic examination of a drop or two of filtered fluid might easily fail to detect the presence of a few spirochetes in the greater, unexamined portion.

Small buds or granules appear to form at the end, or on the side, of some species of *Treponema*, apparently as part of a reproductive cycle. Each bud

or granule appears to give rise to a young spirochete. These granules, or analogous structures, may also form in *Leptospira* and *Borrelia* and account for their filtrability.

#### GENUS SPIROCHAETA

The name of this genus is derived from the name "Spirochaete," by which Ehrenberg, in 1833, designated a very large, spiral and flexible organism found free-living in stagnant water. This organism is now the type species of the genus *Spirochaeta* and is called *Spirochaeta plicatilis*. It is a slender spiral ( $0.5\ \mu$  in diameter) but often attains the relatively enormous length of  $500\ \mu$  ( $0.5\ \text{mm}$ ). The ends are blunt (compare *Treponema*, etc.). Large granules of volutin and fat are present (Fig. 31-1). Motility is by creeping and twisting movements. The organisms may be found in sewage and stagnant water. Pure cultures have been made on medium containing extract of leaves, and on 1.5 per cent agar with red blood cells. *Spirochaeta* are aerobic and grow in a pH range of 6 to 9 at around  $26^{\circ}\text{C}$ . They grow as a thin film on the agar, rarely forming ordinary colonies. They are entirely harmless to man as far as is known.

#### GENUS SAPROSPIRA

This genus resembles the foregoing in that the organisms are found free-living in polluted water, have blunt ends, and are curved and wavy. They are thicker than *Spirochaeta plicatilis*, having a diameter of about  $1\ \mu$ , but are shorter, seldom exceeding  $85\ \mu$  in length (Fig. 31-1). Saprospiras appear to have septate or chambered protoplasm. This could represent separate cells as in *Spirochaeta*, or convolutions of fibril bundles as previously stated. *Saprospira* and *Spirochaeta* may not represent separate genera. Only two or three species are known. Saprospiras have never been cultivated.

#### GENUS CRISTISPIRA

In most respects the cristispiras resemble the saprospiras. They have a wavy arrangement, blunt ends and an apparently septate protoplasm (Fig. 31-1). Motility is active. Their length ranges from 26 to  $120\ \mu$  and their diameter from  $0.5$  to  $3\ \mu$ . The distinct cell membrane stains like chitin; a protozoa-like character. The habitat of *Cristispira* is restricted to the intestinal tract of oysters or to a hyaline structure, called the crystalline style, in the esophagus of these and related mollusks. Cristispiras are distinguished by a *crista*; a sort of keel or membrane, which winds spirally about the organism; one edge free, the other attached to the cell. This structure is suggestive of the undulating membrane seen in one genus of pathogenic protozoa, the trypanosomes. The apparently fibrillar structure of this *crista* has been mentioned. The organisms are entirely harmless. None of the *Cristispira* has ever been cultivated.

#### GENUS TREPONEMA

*Treponema* are slender ( $0.25\ \mu$  to  $0.4\ \mu$  in diameter), and seldom exceed a length of about  $15\ \mu$ . Their size is, therefore, comparable with that of true bacteria. Their cell structure is much less perfectly demonstrable than is that of the larger spirochetes. The organisms have neither *crista* nor septa. The

eight to fourteen spirals found in *Treponema* are close and regular unless the protoplasmic contractions change them. The ends of the organisms are drawn out to extremely fine fibrils. These terminal fibrils have no function in the motility of the organism. Motion may depend on flagella, or flagellum-like fibrils, but the propeller-like action of the spirals when the treponemas rotate doubtless plays an important part.

*Treponema* are not easily stained. Indeed, the first *Treponema* to be described, that causing syphilis (Schaudinn, 1905), was named *Treponema pallidum* because of its pale appearance. Other methods are, therefore, used to demonstrate them microscopically. One is the method of negative staining; another, widely used to examine exudate from lesions of syphilis for diagnostic purposes, is the darkfield apparatus. A third, used mainly by the pathologist to demonstrate spirochetes in infected tissues, is termed "silver impregnation."

**Resistance and Cultivation.** *T. pallidum* is a relatively fragile, highly parasitic organism. It has never been cultivated in artificial media, although it may be maintained alive and virulent for several days in certain artificial media, an important factor in the diagnosis of syphilis. Some similar species (notably the Reiter, Nichols, Noguchi and Kazan strains) have been cultivated, and grow vigorously, but none of these is able to cause infection.

*T. pallidum*, under ordinary circumstances, can survive for only very short periods outside of the tissues of man or experimentally-infected animals. Hence non-venereal infection of man is rare. However, when quickly frozen and maintained at  $-76^{\circ}\text{C}$ , by means of solid  $\text{CO}_2$ , syphilis spirochetes, as well as many other organisms, remain viable and fully infectious for years. The organisms do not long survive ordinary drying. Surface-tension reducers, such as ordinary soap and bile salts, quickly cause lysis of *T. pallidum*. The organism is quickly killed by ordinary disinfectants. In citrated blood stored in "blood banks" the spirochetes quickly die out.

**Syphilis.** Syphilis, caused by *Treponema pallidum*, is primarily a venereal disease, i.e., is transmitted chiefly by sexual intercourse. When so transmitted and when it develops typically it begins, within 2 to 6 weeks after exposure, as a small ulceration on the mucosal surface of the genitalia. The spirochetes rapidly migrate from this to the deeper tissues of the body.

The ulcer increases in size, becoming rather hard and flat. Upon removal of the crust serous fluid oozes from the surface. This, upon examination with a darkfield apparatus, may be found swarming with *Treponema pallidum*. When syphilis is acquired through kissing, the ulcer may appear on the lip. This ulcer, oral or genital, is spoken of as a "primary lesion" or hard chancre (pronounced shank'er). It tends to heal spontaneously due to the development of antibodies after two to six weeks. The victim may believe himself cured. Attempts are made by the local lymph nodes to arrest the migrating spirochetes. The nodes become much swollen and are sometimes called "buboes." Their efforts, unfortunately, are futile.

What really happens is that by this time a certain degree of immunity develops. The *Treponema* have long since migrated from the primary lesion, probably within less than an hour after exposure, and have been carried all over the body. They localize in various organs, particularly the liver, spleen,

walls of arteries, heart, brain, skin and mucosal surfaces, setting up "secondary lesions." These begin to manifest themselves after two to four months. When situated on the skin these often appear as red blotches or an extensive rash and may be very infectious. White patches may also appear in the mouth and genitalia. In such conditions of the mouth kissing of other persons results in infection of the lips, tongue or gums. The teeth may loosen and come out, as well as the hair. After a time (weeks or months) these outwardly visible, *secondary lesions* slowly disappear in great part and the patient may again believe himself cured. Spontaneous cure actually may occur but this is apparently very rare.

In untreated syphilis the *Treponema* usually slowly cause extensive *tertiary lesions* called *gummata* in various internal organs and also on the skin. These tend to heal and form scars as the process continues. The liver becomes damaged and scarred (syphilitic *cirrhosis* of the liver) and bulges appear in the aorta where lesions in the layers of the vessel have weakened it. These bulges are called *aneurysms*. When they burst, death from hemorrhage ensues. Gummata also occur in many of the bones.

The *Treponema* also damage the brain and spinal cord. Various nerve centers are slowly destroyed and characteristic forms of insanity and paralysis result. Death follows, sometimes after a period of many years.

PREVALENCE AND CONTROL. This picture of syphilis is not pleasant and, indeed, the disease is one of the most insidious and dangerous. About twice as many persons die annually of this disease in the United States as die of poliomyelitis. Millions of cases exist which do not come to the attention of the recorder. A million or more new cases develop each year. "V.D." is *not* beaten. It is on the increase because people have been lulled into a sense of false security.

As in the case of gonorrhea, prostitution and sexual promiscuity are the chief means by which syphilis is spread. In spite of renewed efforts by federal, state and local authorities to educate the public to the dangers of syphilis and to enlist the aid of legislatures, medical and civic authorities, and of the people themselves who are endangered by it, many new cases appear each year.

As one great physician has said, "The greatest obstacles to Public Health are the ignorance and indifference of the public!"

SYPHILIS SEROLOGY. The *diagnosis of syphilis*, after the disappearance of the primary lesion in which the spirochetes are demonstrable microscopically, is made by means of serological tests. These have been discussed in Chapter 21. Many non-specific reactions occur.

Three specific antitreponemal tests are now available for the exact diagnostic study of syphilis. These are great steps forward in syphilology. All are dependent on *specific* antibodies against *T. pallidum*. One is the *T.P.I. test*; another, the *immune adherence phenomenon*; the third is a *complement fixation test*, differing fundamentally from the Wassermann test in using extracts of *T. pallidum* as a *specific* antigen (Chapter 21).

#### GENUS BORRELIA

The *Borrelia* resemble the *Treponema* in many respects. However, many *Borrelia* can be stained readily by Gram's method or by means of a special



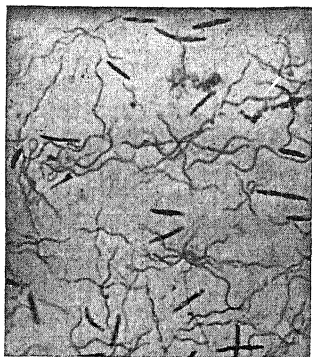


Fig. 31-3. Vincent's angina. Stained smear showing fusiform bacilli and spirochaetal forms. ( $\times 900$ ) (Todd and Sanford.)

polychrome stain made by mixing eosin and methylene blue (Jenner's stain, Wright's stain\*). They often have a less definite spiral form, being more wavy and open, especially in stained preparations. In death they seem to relax and lose their regular, coiled form. They are also somewhat thicker and coarser-looking than the *Treponema*. Cultivation of some has been accomplished although it is not very satisfactory. They grow well in living chick embryos.

Commensal species of *Borrelia* occur, often in large numbers, in the normal mouth (*Borrelia macrodentium*, *B. microdentium*) and on the external genitalia (*B. refringens*). Some of these so closely resemble *Treponema pallidum* in appearance as to create confusion at times in the diagnosis of syphilis by microscopic methods. They appear to be harmless. They are sometimes classed as *Treponema*. Leeuwenhoek probably was the first to observe these.

The majority of the pathogenic *Borrelia*, like *B. recurrentis* and *B. novyi*, are blood parasites causing fever of a relapsing nature (relapsing fever). The spirochetes occur in large numbers in the blood during the numerous febrile relapses characteristic of the disease. They are transmitted by certain ticks and by body lice.

**Trench Mouth.** Some anaerobic species of *Borrelia* are found in the mouth (notably *B. vincentii*) associated with ulcerative conditions ("trench mouth," or *Vincent's angina*). They may be seen readily in gram-stained smears from such conditions, mixed with fusiform bacilli (Fig. 31-3). The name "trench mouth" originated in the frequent occurrence of outbreaks of the disease in soldiers in trenches during World War I. It is presumably transmitted by unclean eating utensils and other articles which carry saliva directly from mouth to mouth. It may be associated with dietary deficiencies or the microorganisms may act merely as secondary invaders of lesions due to other causes: caries, herpes, and so on.

#### GENUS LEPTOSPIRA

*Leptospira* are the smallest of the spirochetes. Their spirals are so fine and so closely wound that, when observed in the darkfield, only the outer curves of the spirals are seen and the organisms appear like strings of minute, illumi-

\* These stains are commonly used for staining blood films to demonstrate the protozoa of malaria. They are available commercially.

nated beads. The leptospiras are further characterized by being bent into a hook at one or both ends (Fig. 31-4). Their motion consists of a writhing and flexing movement and a rapid rotation around the long axis. Their progression from place to place is rapid and can be readily explained on the basis of their screwlike form and their rotation. They may be cultivated readily in relatively simple mineral solutions containing serum. Curiously, no substitute for serum has been found, in spite of diligent search.

Leptospiras are chiefly saprophytic, aquatic organisms which are found in rivers and lake waters, in sewage, and in the sea. Occasionally, they are found in the normal mouth. They may be cultivated in pure culture from some pond or swamp waters by the simple procedure of passing the water through a porcelain or Seitz-type filter (through which they readily pass), and then adding (aseptically) 10% sterile serum; 0.8% salt; and melted agar to yield 0.2%. Incubation is for at least two weeks at 37° C. The organisms are micro-aerophilic and growth appears in a well-defined, greyish zone about 0.5 to 1.0 cm below the surface of the medium.

**Leptospirosis.** There are a dozen or more species (or *sero-types*) of *Leptospira* which are dangerous pathogens. Infection with any of them is properly called *leptospirosis* though special names, of importance historically or otherwise, have been given to certain forms of leptospirosis ("canicola fever," "swineherd's fever," hemorrhagic jaundice or Weil's disease, etc.). Leptospirosis is fairly common, though often wrongly diagnosed.

The various forms of leptospirosis are basically alike though the symptoms vary. In general, the infection is common among certain animals. It is trans-

**Table 18.** *Some Common Leptospiras.*

SPECIES	USUAL ANIMAL HOST	DISEASE*
<i>L. icterohaemorrhagiae</i>	Rats	Weil's disease
<i>L. canicola</i>	Dogs	Canicola fever
<i>L. pomona</i>	Cattle, swine, horses	Swineherd's disease
<i>L. autumnalis</i>	Rats	
<i>L. hebdomadis</i>	Field mice, other rodents	
<i>L. grippityphosa</i>	" " " "	

\* Terms commonly used for various leptospiroses.

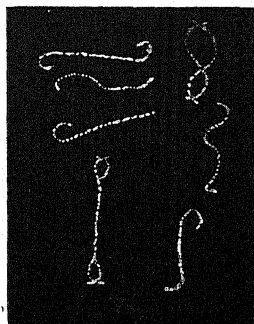


Fig. 31-4. *Leptospira icterohaemorrhagiae*. Appearance of organisms in the darkfield. (× 1000.) (Zinsser, Bayne-Jones, Textbook of Bacteriology, D. Appleton-Century Co., publishers.)

mitted from animal to man through the urine of animals and by anything (water, food, etc.) coming into contact with the skin of, or being ingested by, man. Doubtless the same mode of transmission occurs among animals.

A list of common species of *Leptospira* and their usual animal reservoirs is given in Table 18.

All of the leptospirae are much alike. They are differentiated, mainly by serological means, by special experts.

#### REFERENCES

- Bradfield, J. R. G., and Cater, D. B.: Electron microscope evidence on the structure of spirochaetes. *Nature*, 1952, 169:944.
- Dyar, M. T.: Isolation and cytological study of a free-living spirochete. *J. Bact.*, 1947, 54:483.
- Editorial: Diagnosis of human leptospirosis. *J.A.M.A.*, 1957, 163:188.
- Editorial: Venereal disease control is not automatic. *J.A.M.A.*, 1954, 155:911.
- Editorial: Venereal disease is on the rise. *Am. J. Pub. Health*, 1955, 45:652.
- Expert Committee on Venereal Infections and Treponematoses. World Health Organization Tech. Rep't. Ser. No. 63, 1953. Columbia Univ. Press, New York, N. Y.
- Geiman, Q. M.: Metabolism of spirochetes. *Ann. Rev. Microbiol.*, 1952, 6:299.
- Hyde, H. van Z.: The modern story of yaws. *Am. J. Nursing*, 1955, 55:450.
- Keller, R., and Morton, H. E.: The effect of hand soap and a hexachlorophene soap on the cultivable *Treponemata*. *Am. J. Syph. and Gon. and V. D.*, 1952, 36:524.
- Schlossberger, H., and Brandis, H.: *Leptospira*. *Ann. Rev. Microbiol.*, 1954, 8:133.
- Symposium on the Leptospiroses: Medical Science Pub. No. 1, Army Med. Serv. Grad. School, Washington, D. C., U. S. Gov't Printing Office, 1953.
- Ward, M. K., McDaniel, M. B., Tatum, H. W., Starr, L. E., and Williams, H. R.: An epidemic of canicola fever in man with the demonstration of *Leptospira canicola* infection in dogs, swine and cattle. II. Laboratory studies. *Am. J. Hyg.*, 1956, 64:59.
- White, A.: They said I had syphilis. *Saturday Evening Post*, 1955, 227:(Apr. 30). 27.

## The Spiral, Rigid Bacteria

AT PRESENT, six genera of spirilleae are recognized: *Vibrio*, *Spirillum*, *Cellvibrio*, *Cellfalcicula*, *Desulfovibrio* and *Thiospira*. The name of the first is derived from its rapid motility (from Latin, vibrare, to vibrate); the name *Spirillum* from its form. The names *Cellvibrio* and *Cellfalcicula* are derived from their morphology and their ability to metabolize cellulose. The names *Desulfovibrio* and *Thiospira* are derived from the facts that the organisms are curved and that their metabolisms involve sulfate reduction and sulfur oxidation, respectively.

All of the spirilleae are non-sporeforming, and gram-negative. They are motile by means of polar flagella; hence their inclusion in the family Pseudomonadaceae. They differ from spirochetes (Chapter 31) in being rigid, i.e., not self-flexing. The spirilleae are mainly saprophytic soil and water organisms. Only a few dangerous pathogenic species are found in the group.

### GENUS VIBRIO

This genus includes short rods which are always curved in a portion of a spiral turn (Fig. 32-1). They often remain attached end to end after fission, forming long spirals superficially resembling organisms of the genus *Spirillum* (see Fig. 32-2). The length of the individual vibrios seldom exceeds 10  $\mu$  or their diameter 1.0 to 1.5  $\mu$ . Some are strictly aerobic, some facultative and some strictly anaerobic.

There are numerous "species" or varieties of vibrios, many of which greatly resemble each other, having similar habitats, many physiological properties in common, and sharing several somatic and flagellar antigens. Many are named for the place where they were isolated: *V. danubicus*, *V. gindha*, *V. massauah*, etc.

**V. comma et al.** *Vibrio comma* (discovered in the feces of cholera patients by Koch in 1886), the cause of Asiatic cholera, resembles other vibrios in living for long periods in polluted river and lake waters. (There is no cholera in the Western Hemisphere.) Various strains of *V. comma* are recognized: the Inaba and Ogawa varieties, and others. These differ mainly in antigenic structure and source. Another species possibly of pathologic importance is called *V. proteus*; another the El Tor\* vibrio. This last vibrio is strongly hemolytic

\* El Tor is the name of a town on the western side of the Sinai peninsula.



Fig. 32-1. Electronograph of a characteristic vibrio (*V. fetus*). Note the polar flagella and the curved form which is distinctive of these organisms. These cells, while somewhat shrunken due to necessary drying, show clear differentiation between cell wall and interior cytoplasmic, and possibly nuclear, materials. Original magnification  $\times 30,000$ . (Photo courtesy of Dr. Wayne Binns; in Cornell Veter., 1953, vol. 43.)

when broth cultures are mixed with a suspension of sheep or goat erythrocytes and incubated for 4 to 6 hours (the Greig test). *V. comma* is not hemolytic (Greig negative) under these conditions.

Some vibrios, like *V. metchnikovi*, are pathogenic for guinea pigs and pigeons, which is not true of *V. comma*.

**Isolation of Intestinal Vibrios.** Most intestinal vibrios (and especially *V. comma*) grow at the surface of nutrient liquids in response to need for oxygen. They also tolerate an alkaline reaction (pH 8 to 9) which retards the growth of many of the bacteria associated with vibrios in fecal material (e.g., *Streptococcus faecalis*, *Escherichia coli*, etc.). They also metabolize peptone rapidly. The admixture of beaten, partly digested egg also favors growth. Therefore, very rapid growth occurs at the surface of alkaline-egg-peptone solutions (Dieudonné medium). Transfers from the surface film of such cultures after six to eight hours of incubation often yield almost pure cultures of *V. comma*. Diagnosis is often made by inoculating such medium with stools of a patient suspected of having cholera. This is a good example of *enrichment*.

*Vibrio comma* and its congeners grow well on ordinary laboratory media after initial isolation. The colonies on agar are small, colorless and translucent. Most intestinal vibrios are markedly proteolytic, liquefying gelatin and digesting casein as well as being active in the decomposition of some carbohydrates like dextrose and sucrose.

**Asiatic Cholera.** Cholera is characterized by an intense diarrhea and prostration, due to endotoxins of the vibrios. Great damage also is done by the excessive and rapid dehydration of the patient which is a consequence of the diarrhea. In the acute stage, the mucosa of the large intestine comes away in flakes and the stool, being thin and watery, is described as "rice-water stool."

These flakes of mucosa contain large numbers of microscopically demonstrable cholera vibrios. Cholera is a classical example of what results from lack of sanitation, especially with respect to water supplies. A century or less ago, cholera was to be found in practically every large city in the world, particularly in communities where there was much transient population or in centers for religious, military or other concentrations of large numbers of people, with no effective sanitary provisions with regard to sewage and pollution of water and food. In medieval Europe and later, in America, cholera was an ever-present and often widespread and fatal scourge. It has played a sinister and strictly non-partisan role in many disastrous military campaigns. Because of outbreaks of cholera in the Near East and Eastern Mediterranean areas in 1947, pilgrimages to holy cities from the epidemic areas were stopped. Data published by the World Health Organization set the total number of cases at around 7300, with about 3200 deaths, between September 23 and October 20th. Cholera is found today mainly in the Orient. This is due only to constant vigilance by International Health authorities and in the United States to the activities of federal, state, and local sanitary administrations.

**V. fetus.** This is an economically important species because it causes abortion and considerable reduction in fertility in sheep, cattle and horses and consequent serious economic losses among stock raisers in the United States.

*V. fetus* is a rather highly adapted parasite. It apparently thrives only in the genital organs of male and female domestic mammals (possibly also in wild animals). The infection appears to be transmitted only by coitus. The organisms grow only on very moist organic media in small, translucent, colorless colonies. The slender, curved, individual cells are morphologically much like *V. comma*. The best means of diagnosis is by isolation of the organisms from the animals suspected.

**Anaerobic Vibrios.** Another important group of vibrios comprises strictly anaerobic species found in the human vagina. (If search were made they might also be found, like *V. fetus*, in both sexes.) They appear to cause puerperal (child-birth) infections of the vagina and also may reduce fertility as does *V. fetus* in cattle. Similar (possibly identical) organisms (*V. sputorum*, *V. stomati-*

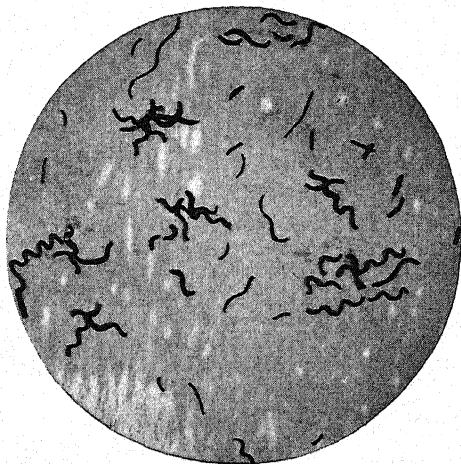


Fig. 32-2. *Spirillum undula* ( $\times 900$ ).

*tidis*) also occur in lesions in the upper respiratory tract and oral cavity. They grow only on moist, rich, organic media, with 15% blood, anaerobically, with a 10% CO<sub>2</sub> content of the atmosphere. Colonies are minute and colorless. They are gram-variable and sometimes gram-positive.

### GENUS SPIRILLUM

This genus contains no species known to be of special interest to the industrialist or the agriculturalist, and only one or two of minor medical interest. All but the last mentioned are harmless saprophytes and scavengers, living, as do most vibrios, in stagnant or polluted water and putrefying materials.

Most saprophytic spirilla are relatively large, ranging from 10 to 50  $\mu$  in length though only 0.5 to 3  $\mu$  in diameter. They are spirally twisted through 1 to 5 complete turns (Fig. 32-2) and are motile by means of tufts of flagella at one or both poles. They usually grow with difficulty on initial isolation.

Some of them have been obtained in pure culture by first preparing infusions of stagnant water, dung or sewage enriched with peptone, meat or fish. After a few days at room temperatures the fluid usually swarms with many sorts of microorganisms. The fluid from such an infusion is sterilized and used as a medium by solidifying it with 2 per cent agar. Poured into Petri dishes, colonies of *Spirillum* can be obtained by inoculation with the unsterilized infusion. Some of the organisms are remarkable in being very sensitive to salt concentrations of even as low as 0.3% NaCl, seemingly preferring distilled (rain?) water. After initial isolation, growth on ordinary medium is relatively easy.

These organisms and many others have also been maintained by a simple and inexpensive procedure for teaching purposes, in a state of "suspended animation" desiccated in a vacuum for several years.

*Spirillum volutans*, one of the largest species, is of interest because of its large metachromatic granules, from which the term *volutin*, in reference to the species, is derived. The motility and form of these spiral organisms is well demonstrated in hanging-drop preparations made from cultures in peptone solution and incubated four days at room temperature. Negative staining is an effective means of making the organisms' arrangement visible. Beautiful demonstrations may also be made with the darkfield apparatus.

*Spirillum minus* and Rat-Bite Fever. *Spirillum minus* causes in man a disease (rat-bite fever), having several of the fundamental clinical features of a typical spirochetal disease (syphilis). *Sp. minus* has never been successfully cultivated. It is gram-negative.

*Sp. minus* occurs in the blood of rats and possibly other animals and is transmitted from them to each other and to man by their bites.

### REFERENCES

- Bhaskaran, K.: Nutritional studies in *Vibrio cholerae*. J. Gen. Microbiol., 1956, 14:XV.  
 Chambers, J. S.: The Conquest of Cholera. The Macmillan Co., New York, 1938.  
 Kuydas, C. D., and Morse, E. V.: A selective medium for the isolation of *Vibrio fetus* and related vibrios. J. Bact., 1956, 71:251.  
 Lewis, I. M.: The genus *Spirillum* Eh'b'g., etc. J. Bact., 1940, 40:271.  
 Myers, J.: Studies on the spirilleae. J. Bact., 1940, 40:705.  
 Seneca, H., and Henderson, E.: Laboratory diagnosis of cholera. Am. J. Trop. Med., 1949, 29:921.  
 Spink, W. W.: Human vibriosis caused by *Vibrio fetus*. J.A.M.A., 1957, 163:180.

## The Aerobic Spore-Forming Rods (Genus *Bacillus*\*)

THERE ARE SCORES of species of bacteria which produce thermoresistant endospores. With only one, possibly two, exceptions, these are all straight, rod forms. The best known non-straight rod form of sporebearer is *Desulfovibrio* or *Sporovibrio* (Chapt. 28). The straight-rod endospore formers are grouped in only two genera: genus *Bacillus* (typically strict aerobes) and genus *Clostridium* (typically strict anaerobes).

Endospores must be distinguished carefully from conidiospores and sporangiospores of molds, ascospores of yeasts and from conidia of the Streptomycetaceae. True bacterial endospores have a high degree of resistance to chemical disinfectants and temperatures commonly used in baking and sterilizing. None of the other spore types approaches this degree of resistance. All of the other forms of spores are means of reproduction. It is generally thought that bacterial endospores are not, because each individual cell produces only one spore. However, some authorities believe some bacteria produce more than one spore per cell.

### GENUS BACILLUS

All of the organisms in this genus are straight, gram-positive (or gram-variable) rods having dimensions ranging around  $1.5\ \mu$  in diameter and  $10\ \mu$  in length. Many species contain numerous fat globules, conspicuous if properly stained. Most species are motile. From the standpoint of cultivation in the laboratory, or growth in natural habitats, they are not fastidious, growing well on simple vegetable or yeast extracts or peptone media. They appear generally to have requirements for complex organic sources of carbon and nitrogen and for various vitamins.

These spore-formers are active and versatile producers of enzymes and consequently can utilize a wide variety of substances as food. A common physiological property is that of hydrolyzing proteins like gelatin, coagulated blood, and dead plant and animal tissues. They also hydrolyze many different carbohydrates and lipids and attack glucosides, alcohols and organic acids.

\* In order to avoid confusion of names, the student is urged to re-read the paragraph on page 12 concerning the use of the terms of "bacillus" and "bacterium."



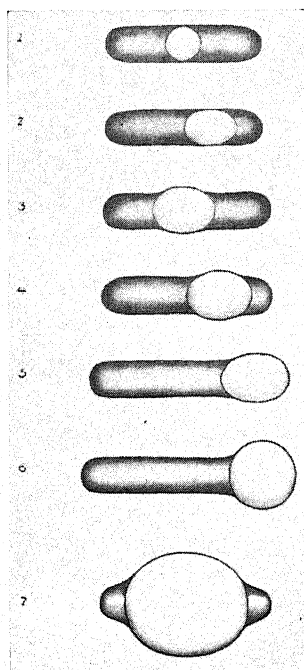


Fig. 33-1. Types of sporulation. 1, spherical; central; cell not swollen. 2, oval; excentric; cell not swollen. 3, Oval; central; cell swollen. 4, oval; excentric; cell swollen. 5, oval; terminal; cell swollen. 6, spherical; terminal; cell swollen. 7, large, oval, central.

At least one species (*Bacillus closteroides*) can utilize carbolic acid. The aerobic, spore-forming bacilli are thus seen to be of great importance as scavengers. Some of their proteolytic and amylolytic enzymes are used in industrial processes (leather, paper, silk, coffee, etc.). Several species are famous and respected in the community because they produce valuable antibiotics: bacitracin, polymyxin, etc. (see Chapter 20). Others are infamous and shunned because they grow in all sorts of valuable commodities (paper, various foods and drugs, wood, leather, etc.) producing spoilage and economic loss to human beings (about whom they care *nothing*!).

There is only one dangerous pathogen for man and animals in the genus: *Bacillus anthracis*, the cause of anthrax.

**Variability.** These organisms are among the most difficult to classify because of the instability of their physiological characters and morphology. It requires the greatest care concerning composition of medium, its pH, temperature of incubation, age of culture and numerous other factors to obtain reproducible results in the study of these species.

**Thermophilic Species.** An interesting aspect of the variability of these bacteria is the development of thermophilic varieties, often designated as separate species. These thermophilic variants (or species) appear to have developed through natural selection of mutants of mesophilic species. They may also represent *adaptive* production of thermostable enzyme systems or other thermostabilizing mechanisms.

**Types of Sporulation and Classification.** Importance has been attached to the form, location and size of spores as a differential character among species of *Bacillus*. These relationships are shown in Figure 33-1. An excellent modern

scheme divides the genus into three main groups on the basis of spore size and form:

1. *Sporangia not definitely swollen*; spores oval to cylindrical; (*B. subtilis*, *B. cereus*, *B. megatherium*, etc.).
2. *Sporangia definitely swollen by oval spores*. Spores rarely cylindrical; (*B. polymyxa*, *B. macerans*, *B. circulans*, *B. brevis*, etc.).
3. *Sporangia swollen by round spores*. (*B. pantothenicus*, *B. sphaericus*, *B. pasteurii*).

**Spore Germination.** The manner in which the spore *germinates* is easily observed and is a particularly constant and distinctive species character. Difficulties due to variation, including complete loss of sporulation, have been mentioned. Sporulation is not always readily seen in cultures of *Bacillus* species. Certain conditions of nutrition must be present for prompt sporulations. Aerobes form spores only aerobically.

Presence or absence of spores is most conclusively demonstrated by means of heat. Growth of a culture after exposure to 90° C for 10 minutes virtually proves the presence of spores.

**Biochemical Characters.** Certain biochemical characters are also of taxonomic value. For example, one system of identification in the genus *Bacillus* is based primarily on the power to hydrolyze urea (urease production), secondarily on sensitivity to acidity and additionally on nutrient requirements, especially of ammonia and certain vitamins.

**Structure of Bacillus.** The electron microscope shows very clearly not only flagella, form and size, but cell wall, protoplast, cytoplasmic membrane and other structures.

A valuable method of studying cell structure is that of embedding bacteria in a material like paraffin (or soft plastic) and making *very thin* slices. These slices are then stained and pictured in the electron microscope. Some remarkable lengthwise cross sections (*sagittal sections*) of some species of bacillus have been thus obtained showing the process of sporulation (Fig. 33-2).

**Distribution.** These bacteria, or their spores, are ubiquitous in soil, dust, and water. Observations of dust storms, and collections of air samples high over the polar oceans have made it clear that spores of these bacteria and other forms of life are carried thousands of miles by currents of air. Darwin, as early as 1831, on board the "Beagle," noted that the dust blown many hundreds of miles out to sea from Africa contained spores. It is not to be wondered at, therefore, that sporeforming, aerobic bacilli are often a source of embarrassment to the unwary bacteriologist who finds them multiplying unwanted in his cultures. They, with molds, are among the most frequent weeds of the microscopic garden. It requires assiduous care and constant vigilance to exclude them.

It was the heat-resistant spores of such organisms that misled Needham and others to support the view that life began spontaneously in the infusions which they *thought* they had sterilized by heating. Even experienced bacteriologists are sometimes embarrassed by the appearance of spore-forming rods in supposedly sterile material or in pure cultures of bacteria. This is usually due to carelessness in the sterilizing room, "short-cuts" in heating processes, etc.

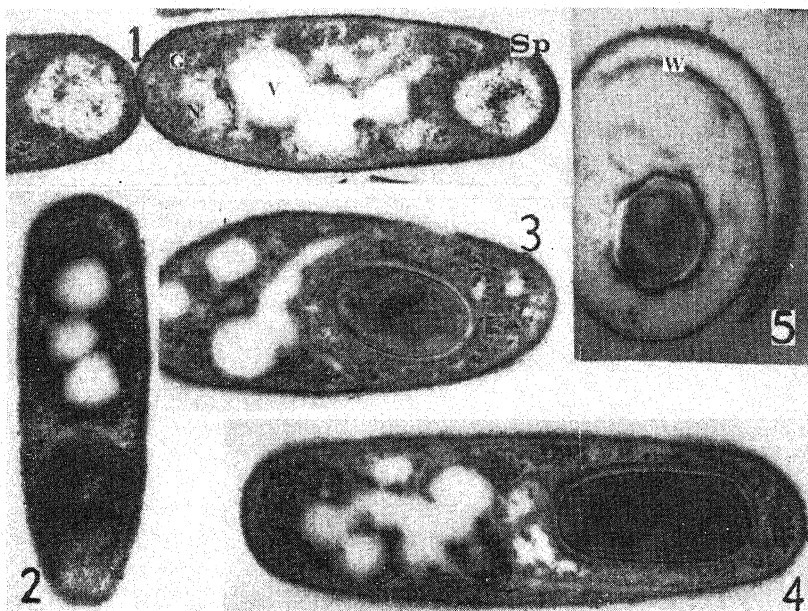


Fig. 33-2. Electronographs of ultra-thin sections of a bacterium (*Bacillus cereus*) showing stages in the formation of a spore. Made with RCA, type EMU, electron microscopes. 1. Two matured cells showing possible nuclear material (N), vacuole-like inclusions (V) and the spore primordium (Sp). The last materializes by differentiation of a mass of the cell content *in situ*. At G are seen granules (these are also seen in the other cells) in the cytoplasm of the parent sporangium. These are of unknown significance but are possibly centers of enzymic activity (mitochondria?). Possibly similar granules are seen inside the spore in 3 and 4. Note that the spore does not, as formerly thought, "grow" from a minute granule such as are seen at G. The primordium of the spore probably represents a collection of nuclear and other essential materials. The physicochemical changes involved in the differentiation of the spore primordium are not yet fully known. 2. The developing spore has changed in its consistency and its opacity to electrons. It is becoming enclosed within a well defined spore coat. 3. Three distinct layers of the spore coat are formed: an inner one probably derived from the spore material; the middle and outer possibly from the cytoplasm of the parent sporangium. At 4 is seen an almost mature spore with three distinct coats. 5 shows a fully mature spore with thick outer coat. The material inside the spore appears to be much less granular (denser?) and of varying opacity to electrons. The broken bacterial cell (sporangium) wall is seen at W. ( $\times 38,000$ ). (Courtesy Dr. G. B. Chapman, in *J. Bact.*, vol. 71, 1956.)

#### BACILLUS ANTHRACIS

Only one species, *Bacillus anthracis*, is very pathogenic to man. It also infects farm animals causing the disease *anthrax*. It differs from most common species of *Bacillus* in being nonmotile. Motility can be induced in it by transduction.

**Anthrax.** This is primarily a disease of farm animals but it is transmissible to man. The name anthrax is from a Greek word meaning boil or carbuncle. In man, the organisms most commonly gain entrance from soil, dust or animal tissues, to the body through a cut in the skin. They first *localize* at the point of entrance, forming a very rapidly progressive, angry, inflamed pustule (*malignant pustule*), which, when well developed, is covered with a

black crust. This pustule teems with anthrax bacilli. It not infrequently heals, but in other cases the bacilli invade the blood stream, multiply enormously, and are spread through all the organs of the body where they tend to form local lesions which serve as further centers for dissemination unless the leukocytes and other defensive mechanisms of the body overcome them. When growing in the body they produce no spores but develop capsules.

Anthrax in farm animals (cattle, sheep) may be prevented by injections of *B. anthracis* bacterins (formaldehyde-killed bacilli) or by injections of spore-vaccines made with *living* spores of graded, attenuated virulence or by the use of serum for temporary, passive immunity. Animals dead of anthrax should be handled with care to avoid contaminating the premises.

#### BACILLUS SUBTILIS

*B. subtilis* is the type species of the genus and is one of the commonest of aerobic spore-formers. It is found in dusty places everywhere and especially in hay. If hay be soaked in warm water for a day or two, the water will be found teeming with organisms of many kinds, among which *B. subtilis* will be prominent. Numbers of other species of *Bacillus* will also be found. These are common contaminants of laboratory cultures.

*Bacillus subtilis* often forms long chains of bacilli sometimes called "streptobacilli." Since the bacilli are motile, such chains swim with a writhing motion. Due to avidity for oxygen, *B. subtilis* and many other species of *Bacillus* grow in a scum or *pellicle* at the surface of fluid media.

Due to its active attack on organic nitrogenous compounds, its cultures smell of ammonia. On slants of potato it grows luxuriantly, with a yellowish or pink color and a warty or vesiculated appearance.

*B. subtilis* is important as the source of the antibiotic, subtilin. Bacitracin is produced from a strain very like *B. subtilis*.

BACILLUS CEREUS, B. MYCOIDES, B. VULGATUS, B. MESPENTERICUS, ETC.

These organisms are very much like *B. subtilis*. All are "hay bacilli," i.e., their spores are found in soil, hay, etc. *B. cereus* forms a spreading, grayish growth on agar, while *B. mycoides* forms very characteristic, mold-like, or nebula-like colonies on agar plates (Fig. 33-3). On potato it produces a whitish, granular growth which becomes brownish later. *B. mycoides* is regarded by some experts as a variant of *B. cereus*.

*Bacillus vulgatus* forms a gummy slime and sometimes causes bread to seem "ropy" by growing in it during damp, warm weather, especially if the bread was made with flour containing many spores of this organism. Its growth on potato is thick, pinkish and wrinkled and turns brown.

*Bacillus mesentericus* is a slime-forming species also; its colonies are sticky and mucoid. On potato it forms a moist-looking, wrinkled scum which turns a dirty brown color later.

*B. vulgatus* and *B. mesentericus* are probably identical.

#### BACILLUS COAGULANS

This organism is of importance as a cause of spoilage of canned foods. It is notable for its ability to grow in acid foods like tomatoes. Since it produces no gas, spoilage ("souring") is not discovered until the container is

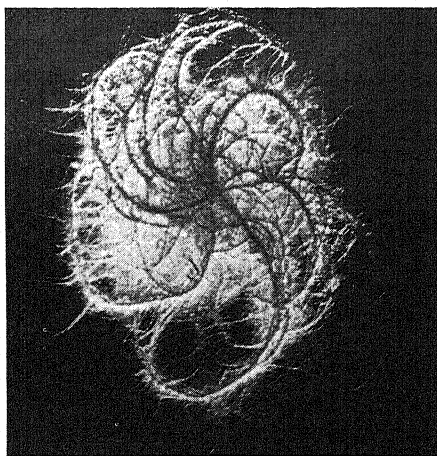


Fig. 33-3. Colony of *Bacillus mycoides* growing on nutrient agar. The curiously curled growth is distinctive of this organism. It is interesting to note that the growth is always curled in a clockwise direction. The reason for this is one of the many mysteries of life. (About life size.) (Courtesy of Dr. F. E. Clark, U. S. Agricultural Research Service, Beltsville, Md.)

opened. It is said to cause "flat sours," because the ends of the can do not bulge as they would if gas were formed under pressure by the fermentation. *B. coagulans* is very heat-resistant and thus often survives commercial processing. It is either a facultative anaerobe or it can grow sufficiently in the small residuum of air enclosed in cans at the time of processing to produce its results. A thermophilic species, *B. stearothermophilus*, very heat-resistant, is also well-known as a nuisance and a source of flat sours in the canning industry.

#### BACILLUS POPILLIAE AND *B. LENTIMORBUS*

**"Milky White" Disease.** Two other species, called *B. popilliae* and *B. lentimorbus*, are now used to combat Japanese beetles. The organisms grow in the "blood" of the larvae and cause the disease commonly known as "milky white" disease. For use against the beetles, the larval juices are dried and ground and mixed with chalk dust or other powder. This is applied to the soil as a spray or dust. Methods of obtaining large numbers of spores of the organisms by using artificial culture media are promising havoc in this branch of "germ warfare" against the nefarious insects. The beetles have disappeared almost entirely in areas where the spores have been applied.

#### BACILLUS ROTANS AND *B. ALVEI*

These species are of especial interest because they produce colonies which move. These motile colonies are discussed in the chapter on Myxobacteriales.

**Foulbrood.** *Bacillus alvei* is of especial importance because it is one of several organisms which cause, or are associated with, a disease (foulbrood) of bees which results in great losses to bee-keepers annually. There are several forms of the disease. American foulbrood is caused by a related organism, *B. larvae*, while *B. alvei* causes European foulbrood. Certain streptococci (*S. apis*) also appear to cause the disease. The larvae of bees contain the infecting organisms in large numbers. This appears to parallel the infection of Japanese beetle larvae by other species of *Bacillus* as noted above.

REFERENCES

- Allen, M. B.: The thermophilic aerobic sporeforming bacteria. *Bact. Rev.*, 1953, 17:125.
- Bornside, G. H., and Kallio, R. E.: Urea-hydrolyzing bacilli. I, II. *J. Bact.*, 1956, 71:627, 655.
- Brown, E. R., Cherry, W. B., Moody, M. D., and Gordon, M. A.: The induction of motility in *Bacillus anthracis* by means of bacteriophage lysates. *J. Bact.*, 1955, 69:590.
- Burdon, K. L.: Useful criteria for the identification of *Bacillus anthracis* and related species. *J. Bact.*, 1956, 71:25.
- Smith, N. R., Gordon, R. E., and Clark, F. E.: *Aerobic Sporeforming Bacteria*. Agr. Monograph 16, U. S. Dept. Agriculture, 1952. Gov't Printing Office, Washington, D. C.
- Stein, C. D.: *Anthrax*. Farmers' Bulletin No. 1736, U. S. Dept. Agric. Gov't Printing Office, Washington, D. C., 1955.
- Steinhaus, E. A.: Living insecticides. *Sci. Am.*, 1956, 195:96.
- Steinhaus, E. A.: *Principles of Insect Pathology*. McGraw-Hill Book Co., New York, 1956.
- Steinkraus, K. H., and Tashiro, H.: Production of milky-disease spores (*Bacillus popilliae* Dutky and *Bacillus lentimorbus* Dutky) on artificial media. *Science*, 1955, 121:873.

## Anaerobiosis. The Genus *Clostridium*

### ANAEROBIOSIS

THE ISOLATION of oxygen by Priestley in 1774 and subsequent observations by Lavoisier about 1775, on the role of oxygen in combustion and respiration, as well as all previous physiological experience, led to the conclusion that free oxygen (air) is necessary to all life. In 1861, however, Pasteur proved that certain yeasts and bacteria could multiply in the absence of air. He devised the term "anaerobiosis" to describe "life without air." This was one of the epoch-making discoveries in biological science. Subsequent studies in the physiology of cells living in situations devoid of free oxygen revolutionized ideas of cell physiology and metabolism.

Since Pasteur's researches many microorganisms capable of living without air have been discovered. These include many common species of bacteria and other familiar forms of life. In a sense, the cells of our own tissues are anaerobic since they have access to oxygen only in combined form (oxyhemoglobin). Yeasts common in baking and brewing grow in the center of rising dough or at the bottom of fermentation vats completely out of contact with air.

**Relations to Oxygen.** As indicated in Chapter 1, microorganisms may be divided into several groups with respect to their relation to free oxygen.

1. *Strictly aerobic* species. These cannot grow without free oxygen to act as final hydrogen acceptor. Their enzyme systems can transfer hydrogen only to free oxygen.

2. *Facultative* organisms. These can use either free oxygen or some other easily reducible substance (e.g., S, C,  $\text{NaNO}_3$ ) as hydrogen acceptor; i.e., they have the *faculty* of growing aerobically or anaerobically. This appears to be usually because they possess both the aerobic as well as anaerobic enzymes.

3. *Strictly anaerobic* species, have two peculiarities: (a) they cannot grow appreciably in the presence of free oxygen (air); (b) their enzyme systems cannot ordinarily transfer hydrogen to free oxygen. They must use other hydrogen acceptors.\*

\* Recent studies support older evidence that some species of strictly anaerobic bacteria can use free oxygen as hydrogen acceptor to a very limited extent. This is probably because such organisms contain large amounts of certain flavine enzymes which can transport some hydrogen to atmospheric oxygen in the absence of the strictly aerobic cytochrome system.

4. *Microaerophilic*. These require limited or lowered oxygen tension but not strict anaerobiosis. The mechanism of this is not fully explained.

$H_2O_2$  AND ANAEROBIOSIS. Now, then, why are strict anaerobes incapable of growth in contact with free oxygen? No fully adequate reply to this question is available. However, there is good evidence that respiration in the presence of free oxygen results in the formation of  $H_2O_2$ , which is very toxic. Thus some strict anaerobes, while possibly capable of some aerobic growth, immediately commit suicide when they attempt it!

"But," you say, " $H_2O_2$  is produced by many *aerobic* bacteria. Why do they not die?" Ah! But these produce *catalase*, an enzyme which immediately decomposes  $H_2O_2$ ! And you (being a well-informed student) say, "True, but many vigorous aerobes do *not* produce catalase. Why does their  $H_2O_2$  not kill them?" So true! We reply, "These are not *sensitive* to  $H_2O_2$ ." Which is a "finisher" worthy of Mark Twain. You would like to ask, "*Why* aren't they sensitive?" and we would say, "Because they have enzyme systems *not* affected by  $H_2O_2$ ." Which is no real explanation and is merely another Mark Twain "finisher" in modern form.\* (By familiarizing yourself with these pros and cons you can astonish and mystify your friends who are not taking microbiology.)

We may list some important or familiar groups of bacteria in relation to oxygen as follows:†

#### 1. STRICT AEROBES

Most species of the genus *Bacillus*

Several species of the genera *Pseudomonas*, *Xanthomonas*

Genus *Brucella* (exc. *Br. bronchiseptica*)

Genus *Azotobacter*

Tribe Nitrobacteriae

Genus *Thiobacillus* (exc. *Th. denitrificans*)

Genus *Acetobacter*

Genus *Mycobacterium*

*Hemophilus pertussis*

Most of family Micrococcaceae

Family Streptomycetaceae

Genus *Nocardia*

Order Myxobacteriales (exc. *Cyto. fermentans*)

#### 2. FACULTATIVE

Family Enterobacteriaceae

Tribe Streptococceae (a few are strict anaerobes)

Genus *Spirillum*

Some of genus *Micrococcus*

Genus *Neisseria*

Genus *Alcaligenes*

\* Especially when we realize that what is demonstrated for one species is not necessarily true for all!

† In each group there are always one or more exceptions. Between the 4 groups there are always "border-line" cases and doubtful species. Hardly anything in biology is absolute; not even the distinction between living and non-living!



## 3. STRICT ANAEROBES

Genus *Clostridium*Genus *Actinomyces*

Tribe Bacterioideae

*Cytophaga fermentans*Genus *Desulfovibrio*

Family Thiorhodaceae

Family Chlorobacteriaceae

*Thiobacillus denitrificans*

## 4. MICROAEROPHILIC

Genus *Leptospira*Genus *Lactobacillus*

## CULTIVATION OF ANAEROBIC BACTERIA

Many types of anaerobic device exist but only one fundamental purpose is involved. This is the removal of free oxygen from the immediate environment of the bacteria, or the maintenance of a low oxidation-reduction potential (presence of a reducing or oxygen-absorbing agent) in the medium itself.

**Oxidation-Reduction Potentials.** Oxidation and reduction can occur in the absence of oxygen and are, as previously pointed out (Chap. 13), basically phenomena dependent on electron transfers. A substance which readily takes up electrons is a good oxidizing agent; i.e., it is readily reduced. Conversely, a good reducing agent is one which readily gives up electrons; i.e., it is readily oxidized. The electron-yielding or electron-accepting potentialities of any given material (a bacterial culture, sewage, etc.), i.e., its oxidation-reduction potential, can readily be measured by thrusting an electrode into it and connecting with a reference cell and a voltmeter (potentiometer) (Fig. 34-1).

**O-R Requirements of Microorganisms.** Anaerobic organisms require low O-R potentials for growth. For example, an O-R potential of  $-0.2$  volt is optimum for the initiation of growth by most species of anaerobes. The effects of various substances added to media to maintain low O-R potentials are shown in Figure 34-2. Once growth has started, the O-R potentials of cultures of all bacteria decline. This is especially marked in cultures of anaerobes since these use every available hydrogen acceptor (tend to release electrons) in their respiratory processes.

## ANAEROBIC METHODS

Anaerobic conditions in culture media may be brought about by three main classes of procedure.

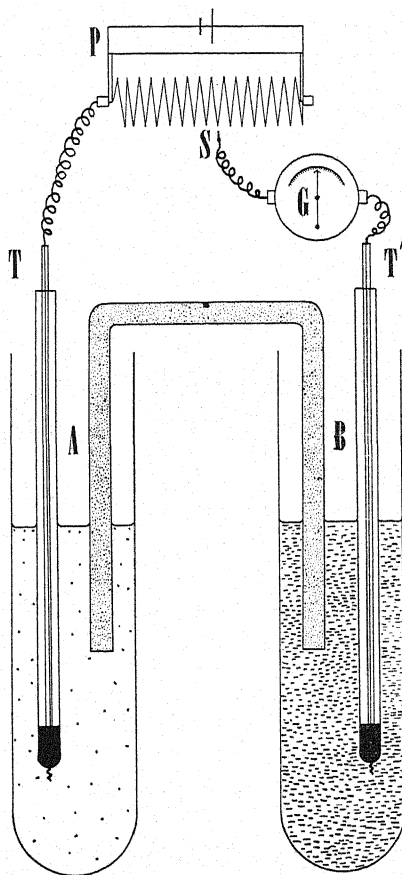
(1) **Chemical.** (a) Cultures may be enclosed in an airtight vessel with sticks of phosphorus or with a freshly made mixture of potassium hydroxide and pyrogallol. These substances absorb large amounts of oxygen and leave mainly the inert gas, nitrogen, and a partial vacuum.

(b) The combustion of small amounts of alcohol or the burning of a small candle in a closed jar will use up some of the free oxygen. Combustion ceases when the carbon dioxide content approximates 10 per cent. This method results in only partially anaerobic conditions. It is widely used to increase

the carbon dioxide content of the atmosphere, a condition favorable to many organisms, both aerobic and facultative, which require  $\text{CO}_2$  for cell synthesis. The reduction of oxygen tension favors microaerophilic organisms rather than strict anaerobes.

(c) A means of *absolute anaerobiosis* is to allow a fine stream of hydrogen to enter a closed vessel, impinging, as it enters, on a small mass of some catalytic agent which causes it to combine with the free oxygen, forming water. A catalyst commonly used is "platinized asbestos" (finely divided platinum deposited on asbestos fibers). This acts very rapidly only when heated. In some types of modern apparatus, the necessary heat is applied to the catalyst by means of an electric current passing through a resistance wire surrounding the mass of catalyst. Danger of explosion due to sparks is eliminated by enclosing the heating element in a gastight tube inside the

Fig. 34-1. In measuring the potential of bacterial culture, *A*, (in this assembly the culture is called a *half-cell*), it is connected, by means of a glass or plastic tube filled with electrolyte (KCl-saturated agar) (stippled), with another half-cell, *B*, (the standard, hydrogen half-cell). The potential of the hydrogen half-cell is fixed and is arbitrarily stated as zero. The potential of the culture is designated as  $E_h$  since it is to be stated in reference to the potential of the standard, hydrogen half-cell,  $E_{st}^*$ . After connecting the electrodes (*T* and *T'*) to the potentiometer (*P*) and galvanometer (*G*) the *EMF* ( $E_1$ ) of the potentiometer (which is indicated on a scale) is varied by means of the slide-wire (*S*) until it just balances the *EMF* of the culture/standard-half-cell system. At this point the galvanometer needle does not swing toward either pole when the circuit is completed.  $E_1$  is then read off on the scale of the potentiometer. Obviously, then,  $E_1 = E_h - E_{st}$  or  $E_h = E_1 + E_{st}$ . Knowing  $E_1$  and  $E_{st}$ ,  $E_h$  is readily calculated.



\* In practice a calomel half-cell (with  $E_h$ , at  $20^\circ \text{C}$  and  $3.5\% \text{ KCl} = 0.254 \text{ volt}$ ) is used in place of the hydrogen half-cell for reasons of convenience and economy. Appropriate allowance is made in the calculations for the voltage of the calomel half-cell.

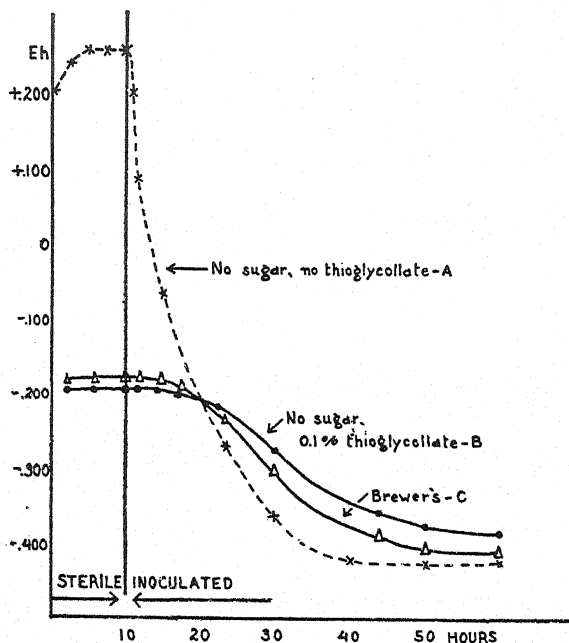
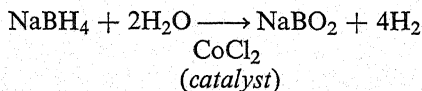


Fig. 34-2. Graphs indicating the O-R potential of growing cultures of *C. perfringens* in peptone-agar medium with and without the reducing agent, sodium thioglycollate (A, B) and in Brewer's medium (C) containing thioglycollate. Note that, before inoculation, medium A had a definitely positive O-R potential. After inoculation, as the *Clostridium perfringens* grew, the O-R potential became rapidly more negative, finally equalling the solutions containing a strong reducing agent. This is characteristic of anaerobic growth in general. It illustrates, in large part, the origin of BOD in sewage (see Chapter 40). Ordinates represent Eh and abscissa time. (Reed, G. B., and Orr, J. H., in *J. Bact.*, vol. 45.)

catalytic mass. A drying agent is enclosed in the vessel to absorb the water that is formed. There is no vacuum, the remaining gas being a mixture of hydrogen and nitrogen.

(2) **Replacement Methods.** Another method of removing oxygen from the atmosphere of closed "anaerobic jars" is simply to flush out all the air with a stream of some inert gas like hydrogen or nitrogen. This leaves an atmosphere of almost pure hydrogen or nitrogen and no vacuum.

The difficulty of obtaining hydrogen from Kipp generators or high-pressure cylinders is readily overcome by placing in the anaerobe jar a tube containing 0.6 gm of sodium borohydride ( $\text{NaBH}_4$ ) in about 40 ml of water, with 0.2 gm of  $\text{CoCl}_2$  as a catalyst. The  $\text{NaBH}_4$  decomposes readily, liberating just enough hydrogen to use up all the oxygen in the jar without generating the dangerous pressure of cylinder gas.



(3) **Oxygen Exclusion Methods.** (a) A simple means of excluding oxygen from single culture tubes is to cover the medium with a layer of sterile petrolatum or mineral oil several cm thick. The medium in the tube under the oil

is boiled or autoclaved and cooled rapidly in ice water (so that oxygen is not reabsorbed) just before inoculation. The heat drives off the dissolved air. If the organism is a gas-former, the gas may blow the petroleum jelly or cotton plug out of the tube. This method is "messy" but effective if carefully manipulated. Withdrawal of material for study from such sealed tubes is awkward.

(b) A simple and effective means is available for obtaining pure cultures of strict anaerobes on the surface of agar plates (formerly possible only by the use of cumbersome, time-consuming and laborious anaerobe jars). The modern method depends on chemical absorption of oxygen from air trapped, by a specially shaped cover, in a very thin layer over the surface of special agar medium in a Petri dish (Fig. 34-3). The oxygen in this air is absorbed by sodium thioglycollate, or some similar compound having an affinity for oxygen, incorporated in the agar. By this means the O-R potential of the medium is held very low and even the most sensitive anaerobes will grow on the agar surface.

(c) Deep tubes of dextrose-infusion agar are also used to cultivate anaerobes. Infusion agar in tubes 8 to 10 cm in depth is melted and cooled to about 50° C. The inoculum is put in and mixed thoroughly. The agar is then made to solidify rapidly in cold water and is incubated. Strict anaerobes will grow only in the depths and will not appear at all within a centimeter or more of the surface. Less strict anaerobes will grow in the depths and will also grow somewhat nearer to the surface, while facultative anaerobes will grow on the surface as well as in the depths. Organisms having a narrow zone of tolerance to both oxygen and strict anaerobiosis (*microaerophils*) may grow in a narrow zone some distance below the surface (Fig. 34-4). Such preparations are often spoken of as "shake tubes" because shaking is used to mix the agar and the inoculum.

**Cultivation of Anaerobes in Media Freely Exposed to Air.** Pure cultures of the strictest anaerobes may be obtained in ordinary media with no precautions as to exclusion of air, provided some substance be added to the media to maintain a low O-R potential.

For example, anaerobiosis in tubes of broth is satisfactory if the medium contains bits of chopped tissue, "cooked meat medium." The tissue acts as a reducing agent. The meat also serves as pabulum for the bacteria. Most anaerobic bacteria grow well in cooked-meat medium.

If the columns of medium in the tubes are 10 to 15 cm deep, all that is necessary is to heat the medium in boiling water for 10 minutes to drive off dissolved air, cool *rapidly*, and inoculate in the depths.

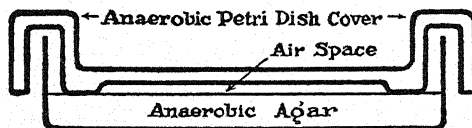


Fig. 34-3. Cross section showing Brewer anaerobic Petri dish cover in use. The anaerobic agar contains the reducing agent, sodium thioglycollate. Note that, at the periphery of the agar surface, the Petri dish cover is in contact with the agar, thus sealing the air space. The thioglycollate absorbs the oxygen from the air space. (Courtesy, The Baltimore Biological Laboratories.)

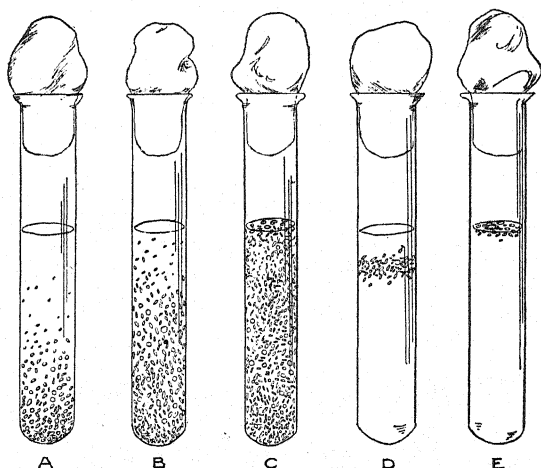


Fig. 34-4. Deep tubes of agar inoculated with bacteria of various oxygen relationships. *A*, fairly strict anaerobe, like *Cl. botulinum*; *B*, less strict anaerobe, like *Cl. perfringens*; *C*, facultative aerobe-anaerobe, like *Esch. coli*; *D*, micro-aerophilic organism like *Br. abortus*; *E*, strict aerobe, like *Pseudomonas fluorescens*.

The addition of sodium thioglycollate (0.1%) or sodium formaldehyde sulfoxalate (0.1%), or both, to dextrose broth or other similar fluids adapts them to anaerobic requirements. The addition of 0.1 per cent agar creates a very slight viscosity which reduces aeration of the solution by convection currents. The use of 0.2 per cent cysteine hydrochloride is as effective, under most conditions, as the reagents mentioned above. The addition of cobalt ions ( $\text{Co}^{++}$ ) to medium as  $\text{Co}(\text{NO}_3)_2$  in concentration of  $4 \mu\text{g Co}^{++}$  per ml also permits aerobic growth of anaerobes by markedly lowering O-R potential. In general, milk, infusion broth and infusion agar with blood, treated with the reducing reagents, are good culture media for anaerobic organisms, since these organisms require media rich in organic matter and having a pH of about 7.2. Chopped brain, fish or other tissues are also often used. The addition of dextrose provides a readily available hydrogen acceptor and source of carbon which promotes the growth of nearly all anaerobes.

### ANAEROBIC BACTERIA

As shown at the beginning of this chapter there are numerous important species of strictly anaerobic bacteria. *Actinomyces* have been discussed in Chapter 30; *Desulfovibrio* and Thiorhodaceae in Chapters 28 and 29, the Chlorobacteriaceae in Chapter 29. There are a few others of lesser significance. Here we may describe briefly the Tribe Bacteroidae and the genus *Clostridium*.

### TRIBE BACTEROIDAE

These bacteria are non-sporeforming, generally gram-negative, small, usually rod-shaped, very pleomorphic. None is motile. There are only two genera: *Bacteroides* and *Fusiformis*.

Some species are normal inhabitants of the upper respiratory tract and genital mucosae. Most species of *Bacteroides* occur in enormous numbers in the intestinal tract. Various species of *Bacteroides* are frequently found apparently as causative agents in lesions of the mucous membranes, in septicemia, in appendicitis, in abscesses of liver, lungs, and other parts of the body. They are often overlooked in diagnostic microbiology because they grow only under strictly anaerobic conditions on media containing blood or ascitic fluid. The colonies are small and colorless. The organisms are fragile, and difficult to maintain alive. The species present in normal feces grow on ordinary laboratory media.

Morphological studies reveal a rather considerable variation in form of *Bacteroides* from global to filamentous. This variation in form appears to be associated with a reproductive cycle like PPLO, involving small forms, like L bodies.

Because of their filamentous variations *Bacteroides* have often been classed with *Actinomyces* and other filamentous bacteria. Some authors include in the tribe, as *Bacteroides fusiformis*, a species of fusiform and filamentous anaerobic bacteria common in the normal mouth. A well known species of these (usually called *Fusobacterium plauti-vincenti*) is associated with *Borrelia* in Vincent's angina or "trench mouth."

Other species of *Bacteroides* frequently occurring in ulcers and abscesses are *B. fragilis*, *B. funduliformis*, and *B. serpens*.

#### GENUS CLOSTRIDIUM

The bacteria belonging to this genus are all obligately anaerobic, gram-positive, spore-bearing rods. Nearly all are motile. They vary somewhat in size and shape, in the manner, say, of cigars, but average around 0.5 by 10  $\mu$  in dimensions. They require complex organic media like cooked-meat medium, glucose-infusion agar or broth, and the like. The group includes the organisms producing tetanus (lockjaw), gas gangrene, and botulism (food poisoning). The majority of clostridia are harmless and helpful saprophytes. Many of them produce enzymes, chemicals and industrial fermentations of great value. All occur widely distributed in the soil. Some of them also live in the intestinal tract of man and animals. They are metabolically active and versatile.

**Clostridium Butyricum.** This is one of the earliest species of *Clostridium* to be studied and is the type species of the genus. It represents the group of industrially important clostridia which are all much alike. In general they are plump, actively motile rods having oval, excentric spores which swell the sporangium. These organisms are widely distributed in the soil. They grow well in media made of dilute molasses or grain extracts, with starch and suitable mineral (and sometimes vitamin) supplements. All have the power of fermenting carbohydrates, with the production of *butyl alcohol*, *ethyl alcohol*, *acetone*, *amyl*, *ethyl*, and *propyl* alcohols and *acetic*, *formic*, and *lactic* acids, *acetone*, *carbon dioxide* and *hydrogen*. The products of fermentation depend on the variety of *Clostridium* used and the condition of the fermentation, i.e., nutrient, pH, temperature, substrate, etc.

**Anaerobic Nitrogen Fixation.** An interesting property of some of these organisms is the power to fix atmospheric nitrogen. That is, they are not

restricted to the use of nitrogen combined in the form of ammonia, nitrates, amino acids, etc., but possess the power to cause free nitrogen of the air to combine in the synthesis of their protoplasm. (See nitrogen cycle, Chapter 40.)

### Pathogenic Clostridia

An important paradox is that although they are highly dangerous pathogenic organisms, *Clostridium botulinum*, *Cl. perfringens* and *Cl. tetani* are not *parasites* but *strict* saprophytes. They grow only on dead matter and cannot invade live tissue. They are all commonly found in the soil, the latter two species especially in animal feces. The spores, consequently, are widespread in manured lands.

**Clostridium Tetani and "Lockjaw."** *Clostridium tetani* is one of the strictest anaerobes. Morphologically, the organism is usually a slender rod ( $0.5\mu$  by 4 to  $8\mu$ ). It bears a spherical spore at the very tip end (terminal) of the rod. The round, terminal spore gives to the organism what has been called a "drumstick" appearance (Fig. 9-10).

*Cl. tetani* gives off a potent exotoxin. Tetanus toxin is particularly active in the motor nerve centers, irritating them so that the muscles connected with them are thrown into a state of violent and continuous contraction (tetanic convulsion or tetanus). The use of antitoxin in the treatment and prevention of tetanus is a classical example of passive immunity and was the first to be discovered (von Behring and Frankel, 1890).

Tetanus organisms gain entrance to the body with dirt or dirty objects when these are forced into the tissues as in gunshot or shrapnel wounds or various accidental means. Under such circumstances some tissue is killed locally by the mechanical injury and, in deep wounds, the low O-R potentials favor growth of anaerobic bacteria. The organism *cannot* invade the *body*, but grows as a saprophyte on the dead tissue in the wound, liberating its deadly toxin which is absorbed by the blood or nerves or both.

Tetanus toxin is one of the most potent poisons known. It requires only about 0.00025 gm of tetanus toxin to kill a man while it requires twenty times as much cobra venom and about one hundred and fifty times as much strychnine to do the same.

**TETANUS IMMUNIZATION.** Alum-precipitated fluid toxoids, in all respects analogous to diphtheria toxoids, are useful in producing active immunity to tetanus (Chapter 22). Protection depends particularly on the action of a primary stimulus consisting of at least one, preferably two, doses of tetanus toxoid a month or so apart. This is now routinely given on entering the Armed Forces. A booster dose is given about a year later. To a secondary stimulus, resulting from entrance of tetanus toxin into the body as the result of a wound, the tissue cells respond quickly with the production of antitoxin. A dose of toxoid in previously-immunized persons is, therefore, often used as prophylaxis in dealing with any fresh wound. It is preferable to avoid the use of serum unless tetanus is imminent. Passive prophylactic antitoxin may then also be used. Even if serum has to be used, toxoid should also be given. Why? The same principles apply in diphtheria (Chapter 35). Combined diphtheria and tetanus immunization employing mixtures of the two toxoids has been found entirely practicable and is routinely given to young infants.

**Clostridium Perfringens.** *Cl. perfringens* is a rather short, thick rod with

rounded ends. It usually grows singly, never in long chains or filaments. It forms oval, central or subterminal spores which do not swell the cell. With four or five relatively unimportant exceptions, it is the only non-motile species in the genus.\*

Certain strains of *Cl. perfringens* are considered by some to be the cause of gastroenteritis when taken in large numbers in food or water. The constant presence of *Cl. perfringens* in feces has led at times to its consideration as an indicator of human fecal pollution when found in water. Because of its pronounced gas-forming powers it is often called the "gas-bacillus."

**STORMY FERMENTATION.** One of its most characteristic cultural reactions is its power to produce a condition in milk called "stormy fermentation." If a column of milk 10 cm deep in a culture tube and containing a little peptone be inoculated with *Clostridium perfringens*, covered with a layer of petrolatum or melted agar and incubated, the lactose is then quickly fermented. Sufficient acid is quickly formed to coagulate the casein. Gas (mainly hydrogen) is then

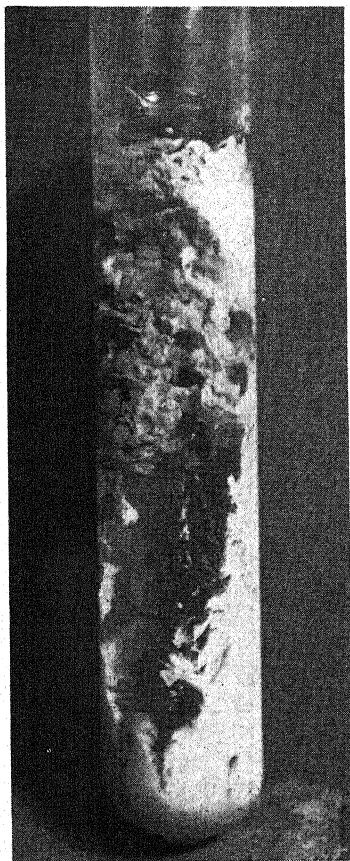


Fig. 34-5. Tube of milk inoculated with *Clostridium perfringens* showing "stormy fermentation." For explanation see text. (Photo courtesy of Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.)

\* In attempting to determine motility of anaerobic bacteria, care must be taken not to expose the hanging drop to the air for more than a few seconds as motility is destroyed by access to free oxygen.





Fig. 34-6. *Clostridium botulinum* cultivated in cooked-meat medium. (× 1000). (Photo courtesy of Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.)

formed in large amounts and the clot of casein is rent asunder, giving the appearance of a very turbulent or "stormy" reaction (Fig. 34-5). Any organism producing sufficient acid to form a solid clot, followed by abundant gas production, could give the same reaction. There are, however, only a few such organisms.

**The Gas Gangrene Organisms.** *Cl. perfringens*, being common in the soil, always accompanies *Cl. tetani* in wounds and, like the latter organism growing saprophytically only on dead tissue, gives off toxins.

**GAS GANGRENE.** In dirty wounds, in addition to *Clostridium perfringens* and *Cl. tetani*, there are nearly always present one or more of about a score of rather similar species of clostridia of the soil such as *Cl. novyi*, *Cl. histolyticum*, etc. Some of these are able to digest dead tissue rapidly, others produce toxins, hemolysins, etc. Some (*Cl. novyi*, *Cl. septicum*) actually invade the blood stream. All of these bacteria are spoken of as gas-gangrene organisms.

In general, the combined unchecked growth of gas gangrene organisms in dirty wounds such as crushed limbs, shell wounds, nail punctures, etc., where there is much dead tissue, produces a rapidly fatal condition known as gas gangrene. It used to be much feared by soldiers wounded on the battlefield.

**Clostridium Botulinum.** This is one of the two organisms (*Micrococcus pyogenes* and *Cl. botulinum*) causing food poisoning. *Cl. botulinum* is a strict anaerobe and forms large, oval spores in a subterminal position, often giving the sporulating rod a shape that is said to resemble a snowshoe (Fig. 34-6). These spores are very heat-resistant. Like the other clostridia, *Cl. botulinum* is widely distributed in the soil.

It derives its name from the Latin word for sausage (*botulus*). It was given its name because it was first found in sausages which were the cause of fatal food poisoning (botulism). The interior of a sausage (or canned foods if not sterilized) obviously presents an ideal place for the growth of anaerobes (Chapt. 43).

#### REFERENCES

- Brewer, J. H., Heer, A. A., and McLaughlin, C. B.: The use of sodium borohydride for producing hydrogen in an anaerobe jar. *Appl. Micr.*, 1955, 3:136.

- Cockburn, W. C., and Vernon, E.: Food poisoning in England and Wales, 1954. Monthly Bull. Ministry of Health and the Public Health Lab. Serv., (London) 1955, 14:203.
- Dack, G. M.: Food Poisoning. 3rd ed. University of Chicago Press, Chicago, Ill., 1956.
- Dedic, G. A., and Koch, O. G.: Aerobic cultivation of *Clostridium tetani* in the presence of cobalt. J. Bact., 1956, 71:126.
- Dolman, C. E.: Additional botulism episodes in Canada. Can. Med. A. J., 1954, 71:245.
- Editorial: Tetanus immunization. J.A.M.A., 1956, 161:883.
- Hewitt, L. F.: Oxidation-reduction potentials in bacteriology and biochemistry. 6th ed. London County Council, London, 1950.
- Lev, M.: Aerobic cultivation of *Clostridium tetani*. J. Bact., 1956, 72:718.
- McClung, L. S.: The anaerobic bacteria with special reference to the genus *Clostridium*. Ann. Rev. Microbiol., 1956, 10:173.
- Pulaski, E. J.: Medical progress: war wounds. New England J. Med., 1953, 249:890, 932.
- Various Authors: Symposium on Anaerobes. J. Appl. Microbiol. (London), 1956, 19:1.

## Family Corynebacteriaceae; Family Lactobacteriaceae

### THE CORYNEBACTERIACEAE

THE NAME of this family is derived from Greek words meaning "club-shaped bacterium," referring to the club-shaped cells often formed by some species, notably *Corynebacterium diphtheriae*.

The family contains three genera: the genus *Corynebacterium*, of which *C. diphtheriae*, the cause of diphtheria, is type species; the genus *Listeria*, of which *L. monocytogenes*, a motile "diphtheroid" and the cause (?) of "glandular fever" (more properly, listeriosis) in man and animals, is the only species; and *Erysipelothrix*, of which the cause of swine erysipelas, *E. rhusiopathiae*, is the type species.

All are gram-positive (or gram-variable) non-sporeforming, non-motile (except *Listeria*) generally short, plump, rods ranging around 1.0 by 8.0  $\mu$  in dimensions and exhibiting various degrees of pleomorphism, especially in *Corynebacterium* and *Erysipelothrix*. Most of the species in this family grow best aerobically, on infusion media at 30 to 40° C, pH 7.0 to 8.0, particularly if serum (or blood) and dextrose are added. Except for a few parasitic species, they are saprophytes, widely distributed in the environment: dust, dung, dairy products, on the skin, in decaying organic matter, etc. These are frequently encountered as contaminants in laboratory cultures.

### THE GENUS CORYNEBACTERIUM

There are several animal pathogens and numerous harmless saprophytes in this genus. Some motile species have been described, but these are not true corynebacteria. To the farmer and veterinarian important species are: *C. pyogenes*, which is common in purulent lesions of cattle, swine and sheep; *C. equi*, causing pneumonia in foals; and *C. renale* which causes a necrotic disease of the urinary tract in cattle. In addition, the genus includes a number of important plant pathogens, like *C. michiganense*, the cause of tomato canker, and *C. insidiosum*, cause of a destructive disease of alfalfa.

Differentiations between species of corynebacteria are commonly made on the basis of fermentation reactions, pigment formation, gelatin liquefaction and morphology. In general, they are not very active in attacking carbohydrates, proteins or fats.

From the standpoint of human health the most important species is *C. diphtheriae* although the microaerophilic *C. acnes*, implicated as a cause of acne (so often a temporary thorn in the flesh of all who are young and beautiful), has an importance in human happiness.

*C. diphtheriae* is usually distinguishable by: (1) great variation in length of the cells, from coccoid to spindles or clubs 10 to 15  $\mu$  in length; (2) great variation in shape, from club-shaped to sperm-like, needle-shaped or boomerang-shaped forms; (3) conspicuous intracellular granules, bars and masses. These have been regarded as nucleus-like structures but are more generally regarded as volutin (Fig. 35-1); (4) irregularity of arrangement; (5) marked affinity of the volutin in the cells for methylene blue. The result of this is that the granules, bars, or the entire cell (depending on the distribution of the volutin in the cell) stain a *very intense blue* or *metachromatic* (red) color.

**Diphtheroids.** Species of corynebacteria having a more regular length, form and arrangement, which differentiate them from *C. diphtheriae*, are spoken of collectively as "diphtheroids" (Fig. 35-2).

A number of similar gram-positive rods, now separated in the genera *Microbacterium* (lactic acid producer), *Butyribacterium* (butyric acid producer), and *Propionibacter* (propionic acid producer), (Tribe Lactobacilleae) are closely related and similar to corynebacteria in a number of respects. They are discussed farther on in this chapter.

The colonies of corynebacteria on solid media, such as blood infusion agar, are generally white or yellowish, opaque, round and range in diameter from about 1 mm to 3 or 4 mm. They are usually soft and butyrous but some

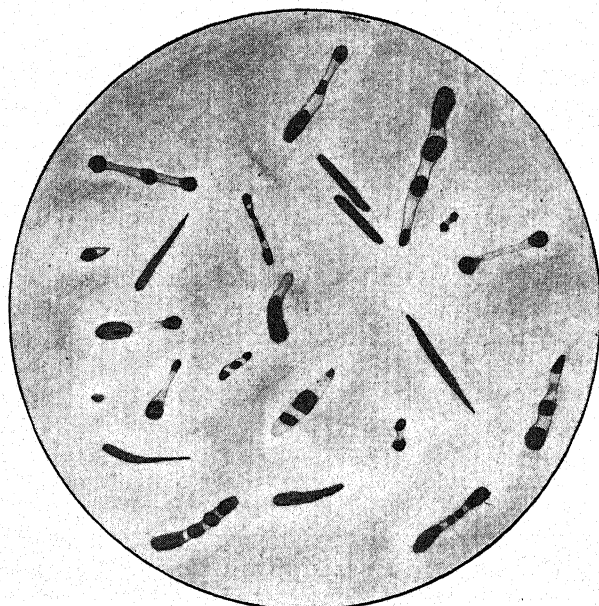


Fig. 35-1. *Corynebacterium diphtheriae*. These have been stained with Loeffler's alkaline methylene blue solution. Note the great variation in length, the pleomorphism and the volutin arranged as bars and granules and sometimes filling the entire cell. (2500  $\times$ ).

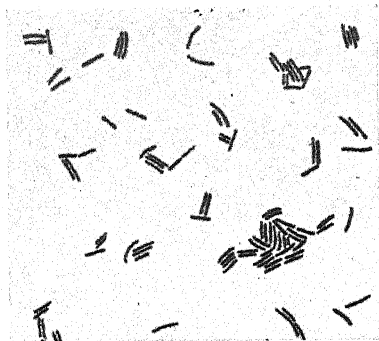


Fig. 35-2. A typical diphtheroid—*Corynebacterium acnes*. (Ford, Textbook of Bacteriology.)

species form irregular and brittle colonies. Many species form brilliant pigments.

We may well give some attention to diphtheria because it illustrates a number of important principles of medical microbiology and immunology. What is true of one infection is often, in whole or in part, true of other infections. Formerly a widespread and fatal scourge, diphtheria now kills less than 3000 persons in the United States annually.

### Diphtheria

Diphtheria is a specific disease due to *Corynebacterium diphtheriae*. The organisms are transmitted in the same manner as others causing respiratory disease. Healthy carriers are not uncommon (about 1 per cent of the population) and are doubtless sources of cases. The bacteria establish themselves on the mucous membrane of the throat and nose and excrete exotoxin.

**Toxin Production by *Corynebacterium Diphtheriae*.** *C. diphtheriae* secretes one of the most powerful biological poisons known. When the organisms grow on the tonsils this poison is absorbed by the blood and damages heart, kidneys, adrenals and nerves and may cause death unless antitoxin is (a) already present or (b) is developed rapidly by the cells of the patient, or (c) is injected into him from some outside source. The toxin is also very poisonous to rabbits, mice, guinea pigs and to chicks and other birds.

*Corynebacterium diphtheriae* is a classical illustration of virulence depending almost entirely on toxigenicity. This organism, although often growing extensively on the surfaces of the respiratory tract, has little ability to invade the tissues beyond the mucous membrane.

**Immunity to Diphtheria.** Most persons more than 15 years of age possess a natural, *active* immunity probably through *subclinical* attacks during earlier childhood, and retain their immunity throughout life, probably as a result of repeated reinfection. Their blood usually contains a small amount of antitoxin and this helps combat ordinary infection. In addition, as pointed out in the chapter on Allergy, *natural* immunity includes a *tissue-reactivity* or binding power for the *bacilli* and probably *toxin*, which is a pre-antitoxin line of defense.

✓**THE SCHICK TEST.** It is possible to determine whether or not a person's blood contains sufficient antitoxin to help protect him. Of the amount of diphtheria toxin which takes four days to kill a small guinea pig (1 *minimal lethal dose*, as it is called), one fiftieth is injected *into* the skin of the person

whose immunity is to be tested. If the person's blood contains a sufficient amount of diphtheria antitoxin to protect him (about 0.01 unit\* per cubic centimeter of serum), nothing happens. If his blood contains too little antitoxin, a small red spot appears at the site of the injection and remains for some days. This is called a positive *Schick test*, the test having been devised by the Austrian physician, Schick. It is perfectly harmless and may do some good by stimulating antibody production.

If the natural immunizing processes of often-repeated natural reinfection, which depend on prevalence of healthy carriers of virulent organisms, are diminished, then many persons may fail to be naturally immunized. Their Schick test may remain positive. To supplement natural immunization processes artificial active immunization is widely used.

**ACTIVE IMMUNIZATION TO DIPHTHERIA.** Some children giving a positive Schick test may contract diphtheria. Whether they do or not depends on dosage of infective bacilli, natural resistance and other factors. For safety, they should be artificially, actively immunized sufficiently so that they give a



Fig. 35-3. Immunization to diphtheria. This picture shows the simplicity of the procedure. It is of great historical interest, since it is a portrait of the late Dr. William H. Park, long Bacteriologist for the City of New York, who introduced diphtheria immunization to this country (right); and Dr. Schick, the famous Austrian physician who devised the Schick test for immunity to diphtheria (standing, white gown).

\* A unit, roughly, is sufficient antitoxin to neutralize a little over 100 minimal lethal doses of toxin.

negative Schick test. Any physician or health department will do this on request (Fig. 35-3). The process requires only two injections of toxoid, alum-precipitated, given 4 to 6 weeks apart. The toxoid is often mixed with tetanus toxoid and pertussis (whooping cough) vaccine, with excellent results in respect to all three diseases. Since this immunity wears away in time, it is customary to reimmunize with repeated small doses ("booster doses"), a year later and then every two to three years.

Adults not infrequently give positive Schick tests, but do not have diphtheria nearly so often as children, from which it may be inferred that such persons have: (a) binding power of their tissues as a result of natural immunization, and (b) an enhanced, allergy-like ability to respond to infection very rapidly by producing antitoxin in their blood. Both types of response result from a previous slight infection or *primary stimulus*.

**PASSIVE IMMUNIZATION TO DIPHTHERIA.** A person ill with diphtheria, or a child exposed to the disease by living in the same house with such a patient, may have immediate need of antibodies to combat the disease or ward off infection. Endangered persons may, under special circumstances to be judged by the physician, receive immediate protection through injections of serum which contains large quantities of antitoxin.

**Laboratory Methods for the Study of Diphtheria.** Bacteriological procedures for diagnostic study of diphtheria may be grouped under three general headings as follows:

1. **INITIAL THROAT CULTURE.** The commonest cultural procedure consists in rubbing a sterile "swab" (a wooden applicator, tipped with cotton) over the infected or suspected tonsils and nasal mucosa, and then rubbing the swab over the surface of sterile, coagulated serum (Löffler's medium\*) or sterile coagulated eggs (Pai's medium†). After 2 to 24 hours' incubation at 37° C a methylene-blue-stained smear is made from the mixed growth on the surface of the medium. Organisms having the characteristic morphology of *Corynebacterium diphtheriae* are sought among the myriads of other organisms present. Considerable experience is necessary for this work for it is in such material that one often encounters diphtheroids.

2. **ISOLATION.** There are some organisms morphologically indistinguishable from *C. diphtheriae* which are perfectly harmless. In order to be sure that diphtheria-like organisms found in the throat are really virulent, it is sometimes necessary to isolate them in pure culture and test their virulence. This is commonly done by injecting rabbits (Fig. 35-4).

3. **THE IN-VITRO TEST.** As a result of observations by Ouchterlony, Elek, and others, virulence (toxigenicity) of *C. diphtheriae* and some other toxigenic organisms may be demonstrated, *without using animals*, by an in vitro toxigenicity test. The test illustrates a very important principle in immunology. It is described in detail in Chapter 21. The in vitro plates may also be inoculated directly with the initial swab from the patient, or with colonies picked from the tellurite plate. This often saves 24 to 48 hours of the time required to isolate pure cultures.

\* **Löffler's medium.** To 3 parts of serum add 1 part of 1 per cent dextrose broth. Tube and sterilize 1 hour in a slanting position without releasing steam or air pressure.

† **Pai's medium.** For the serum in the above, substitute whole hen's eggs, and for the broth substitute distilled water. Mix well and sterilize in the same manner.

**TYPES OF CORYNEBACTERIUM DIPHTHERIAE.** In 1931 attention was called to several different types of *C. diphtheriae*. Because of the rather constant presence of one type in severe (grave) cases of the disease it was thought to be the cause of severe cases and was called the *gravis* type. Another type, found at that time in less severe cases and in many healthy carriers, was thought to be less dangerous and was called the *mitis* type. A third type found in moderately severe cases, and having some of the properties of both *mitis* and *gravis* types, was called the *intermedius* type. In Baltimore, during an epidemic in 1944, a fourth type was found, which was named the *minimus* type. The differential characters of these types are shown in Table 19. Experience in the United States indicates that none of these types has any special clinical significance, though European observations support the view that they may, in some instances.

**Alterations in Virulence of *C. Diphtheriae*.** Wholly avirulent (atoxigenic) cultures can suddenly acquire high grade toxigenicity (virulence). The change is brought about by the action of certain bacteriophages (see Transduction, Chapter 15).

#### GENUS LISTERIA\*

The organisms of this genus are in most respects much like *Corynebacterium pseudodiphtheriticum* and other diphtheroids except that they are more regular in form and *motile* by means of peritrichous (or polar?) fla-

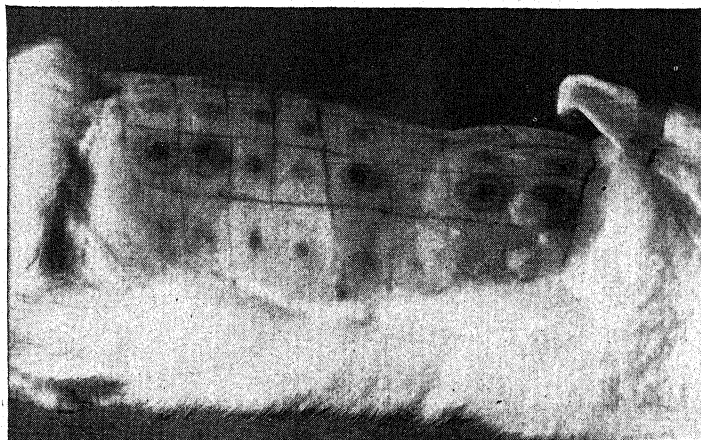


Fig. 35-4. Intradermal tests for toxigenicity in a rabbit. The cultures to be tested were injected in 0.2-ml. doses into the squares marked on the skin. Five hours later, the rabbit received 1000 units of diphtheria antitoxin intravenously. The test cultures were then immediately reinjected, each in a square adjacent to the corresponding initial injection. The large, circular dark areas are zones of necrosis and inflammation produced in the skin during the five hours *before* diphtheria antitoxin was injected into the ear vein. After injection of antitoxin the same cultures, each injected into a square below the first injection, failed to produce any necrosis and only an insignificant amount of inflammation, showing that the necrosis could be prevented by diphtheria antitoxin. Since the antitoxin is specific in its action, this proves that the bacilli being tested were toxigenic (virulent) *C. diphtheriae*. (Specimen prepared by Miss Elizabeth O. King, Photo courtesy U. S. Public Health Service, Communicable Disease Center, Atlanta, Ga.)

\* Named for Lord Lister, British scientist.



Table 19. *Cultural Properties of Types of C. Diphtheriae.\**

TYPE	FERMENTATION OF				FORM AND DIAMETER OF COLONY†	HEMOLYSIS‡	PELLECLE FORMATION	FINAL pH OF BROTH
	SUCROSE	DEXTROSE	GLYCOGEN	STARCH				
Gravis	—	+	+	+	Low; conical; irregular margin and surface; (rough); 2-4 mm.	—	+	7.5-8.4
Mitis	—	+	—	—	Doomed; smooth, regular; 1-2 mm.	+	—	6.5-7.4
Minimus	—	—	—	—	Surface and margin smooth; very minute; usually flat; 0.05 to 0.5 mm.	—	—	6.5-7.4
Various others not named	— +	— +	— —	— —	Usually like mitis type	— +	— +	Variable

\* Any of these may be toxigenic or atoxigenic.

† On heated blood agar with tellurite.

‡ Mix 0.5 ml 48-hour broth culture with 0.5 ml 2 per cent human erythrocyte suspension.

|| In absence of dextrose, after 5 days at 37° C.

gella; a notable exception among the gram-positive, non-sporeforming rods (see Table 28). *Listeria* grow readily on blood and serum media, at 37° C. They are facultative.

**LISTERIOSIS.** *Listeria* appears to be regularly pathogenic for man and lower animals, causing a febrile disease characterized especially by swollen lymph nodes and the appearance in the blood of large numbers of white cells called *monocytes*; hence the species name: *Listeria monocytogenes*.

#### GENUS ERYSIPELOTHRIX

Every farmer who has raised many hogs for market probably knows about swine erysipelas. Only the fortunate farmer has escaped the infection himself. *Erysipelothrix rhusiopathiae*,\* the causative organism, is widely distributed in soil, dung, dust and sewage and can infect sheep, birds, rodents, fish, etc.

*E. rhusiopathiae* in many respects resembles the diphtheroids. However, it is often pleomorphic and filamentous, like lactobacilli, especially in the R phase. Because of its tendency to filament formation it has sometimes been classified in the Actinomycetales. It is facultatively aerobic. It is quite resistant to drying and outdoor conditions generally and, therefore, can persist stubbornly in the dust and dirt of animal pens, buildings and vehicles where infected animals have been. It is transmitted by inhalation and ingestion of infected dirt and by way of cuts and scratches.

**Swine Erysipelas.** Swine erysipelas is the commonest form of disease produced by *E. rhusiopathiae*. It is usually slowly progressive, though the infection at times is highly and rapidly fatal in swine herds (and very costly to stock raisers). It can be isolated on blood agar from the lesions of infected animals. Because the reddish skin lesions are often roughly diamond-shaped swine erysipelas is sometimes called "diamond disease."

#### THE FAMILY LACTOBACTERIACEAE

These organisms are discussed here because they have many points of resemblance to the *Corynebacteriaceae* and also to some of the spherical bacteria (*Streptococcus*, *Leuconostoc*). They are of great importance in various industries. The family *Lactobacteriaceae* consists of two tribes: Tribe I *Streptococceae*; and Tribe II *Lactobacilleae*. The tribe *Streptococceae* are described in the next chapter, the *Lactobacilleae* in this chapter.

#### TRIBE LACTOBACILLEAE

The tribe *Lactobacilleae* comprises four genera: (1) *Lactobacillus*; (2) *Microbacterium*; (3) *Propionibacterium*, and (4) *Butyribacterium*.

All of these organisms are gram-positive, non-sporeforming, non-motile, microaerophilic and facultatively aerobic. All are aciduric, several are acidophilic, and nearly all are thermoduric and/or thermophilic. Morphologically they are rods, generally resembling the diphtheroids. However, *Lactobacillus* is much more pleomorphic. For example, under some conditions of growth

\* *Erysipelo* is from Greek words meaning red skin; the suffix *thrix*, from a Greek word meaning thread. Of the species name, *rhusio-* is from the Greek for reddish in color; *pathiae* is from the Greek for disease producer. The whole name literally translated, therefore, means "Erysipelas-thread, reddish-disease producer."

*Lactobacillus* forms rods so short as to be coccoid. When in chains they closely resemble streptococci. At other times they form long chains of slender bacilli (*streptobacilli*) or long filaments resembling *Erysipelothrix*.

### Genus *Lactobacillus*

These important organisms thrive in sour milk, sauerkraut, pickle vats, etc., *after* these products have been made moderately acid by the growth of other organisms. Lactobacilli are nutritionally fastidious organisms, requiring complex organic media with vitamins. In the laboratory they grow poorly on ordinary agar; heavy inoculations are necessary to produce even a few small, translucent, delicate colonies. They grow best on tomato-juice agar and whey agar at about 25° C. Their natural habitat is on plants, in dairy products, soil, dung, etc. They are vigorous fermenters, producing much lactic acid. They are generally harmless and their acid production is of use in food-producing industries.

The genus may be divided into convenient groups as follows:

#### GROUPS OF LACTOBACILLUS

- I. Homofermentative.** (Products of fermentation are almost exclusively lactic acid: 98–99%; never CO<sub>2</sub>).
- A. Mesophilic (grow at 12° C; not at 45° C) (Sometimes grouped as *Streptobacterium*).
1. *L. plantarum* (common in dairy products and fermented vegetable products).
  2. *L. casei* (one of the most common lactobacilli in dairy products).
  3. *L. bifidus* (common in feces of breast-fed infants; anaerobic; shows Y, L and T forms).
- B. Thermophilic (grow at 45° C; not at 20° C) (Sometimes grouped as *Thermobacterium*).
1. *L. lactis* (one of the most common lactobacilli in dairy products).
  2. *L. acidophilus* (common in milk and in intestinal tract of man and animals; not sensitive to surface tension reducents such as bile).
  3. *L. bulgaricus* (common in vagina and sour milk; not in intestinal tract; is sensitive to surface tension reducents such as bile).
  4. *L. caucasicus* (found in kefir and some cheeses; see Chapter 42).
- II. Heterofermentative.** (Products of fermentation include lactic and acetic acids, alcohol and CO<sub>2</sub>) (Sometimes grouped as *Betabacterium*).
- A. Mesophilic
1. *L. brevis*
  2. *L. pastorianus*
- B. Thermophilic
1. *L. fermenti*
- widely distributed in nature. Less desirable in fermented food products because of gas, alcohol and "off" flavors.

Both homofermentative and heterofermentative species are used extensively in food manufactures (dairy products, sauerkraut, etc.). (See Chapters 43 and 44.)

**ORAL LACTOBACILLI.** A good many of these species, and some others (*L. salivarius* and *L. cellobiosus*) are often found in the oral cavity, especially around the teeth. With aciduric streptococci they are important as causes of dental caries, due to the acidity which they produce.

### *Genera Microbacterium, Butyribacterium and Propionibacterium*

These three genera comprise several species of gram-positive rods much like the saprophytic diphtheroids.

Genus *Microbacterium* includes two species: *M. lacticum* and *M. flavum*, common in the same habitats as many saprophytic corynebacteria and lactobacilli. They have little known significance except as saprophytes. They produce lactic acid during fermentation.

Genus *Butyribacterium* includes only one species, closely resembling saprophytic diphtheroids. It is heterofermentative, producing butyric acid and CO<sub>2</sub>.

Genus *Propionibacterium* is somewhat more pleomorphic than the two foregoing genera; more like *Corynebacterium xerose* or *Erysipelothrix*. Otherwise it shares the general characters of saprophytic diphtheroids. Some species produce reddish pigments.

Of some 10 or 12 species of *Propionibacterium* several are commonly found in various hard cheeses. During the ripening of these *Propionibacterium* contributes flavors due to propionic acid; for example, the distinctive sweet and bitter flavor of Swiss cheese is partly due to propionates. The eyes of Swiss cheese are due to CO<sub>2</sub> and air. The origin of the eyes in these cheeses is not fully explained, but *Propionibacterium* is known to produce CO<sub>2</sub>, being heterofermentative. Pure cultures of *Propionibacterium* are added to milk in the manufacture of Swiss, Münster, and similar cheeses.

### THE "LACTIC BACTERIA"

The Lactobacillae are often found in dairy products, dung, soil, plants, etc., in company with well-known species of Streptococcae: *Streptococcus lactis*, *Leuconostoc mesentericus*, etc. All have similar physiology, habitat, and ecological relationships. All except *Propionibacterium* and *Butyribacterium* form considerable amounts of lactic acid during fermentation, and can thrive in considerable concentrations of this acid. For this reason they are collectively spoken of as "lactic-acid bacteria" or the "lactics."

All of the lactic acid bacteria are of great industrial and agricultural importance, since they (with other microorganisms) are concerned in the souring of milk and cream for butter and cheese making; the preparation of yoghurt and other fermented milk products; the curing of ensilage; the manufacture of lactic acid and of sauerkraut; and the fermentation of pickles, olives, citrons, etc. They are more fully discussed later on.

### REFERENCES

- Davis, G. H. G.: The classification of Lactobacilli from the human mouth. J. Gen. Microbiol., 1955, 13:481.

- Diagnostic Procedures and Reagents. 3rd ed. Chapter on Diphtheria. Am. Pub. Health Ass'n., New York, 1950.
- Frobisher, M., Jr., and Parsons, E. I.: Studies on type-specific immunization with somatic antigens of *Corynebacterium diphtheriae*. Am. J. Hyg., 1950, 52:239.
- Frobisher, M., and Parsons, E. I.: Further studies of tellurite plating media for *Corynebacterium diphtheriae*. Am. J. Pub. Health, 1953, 43:1441.
- Hagan, W. A., and Bruner, D. W.: The Infectious Diseases of Domestic Animals. 2nd ed. The Comstock Pub. Co., Ithaca, N. Y., 1951.
- Jensen, H. L.: The coryneform bacteria. Ann. Rev. Microbiol., 1952, 6:77.
- Kolb, R. W., Branham, S. E., and Riggs, D. B.: Comparison of the guinea pig and chick in evaluation of diphtheria toxin for the Schick test. Appl. Micr., 1955, 3:241.
- Lautrop, H.: On the existence of an antibacterial factor in diphtheria immunity. Acta Path. et Micr., Scandinavica, 1955, 36:274.
- Parsons, E. I.: Induction of toxigenicity in non-toxigenic strains of *C. diphtheriae* with bacteriophages derived from non-toxigenic strains. Proc. Soc. Exp. Biol. and Med., 1955, 90:91.
- Parsons, E. I., Frobisher, M., Moore, M., and Aiken, M. A.: Rapid virulence test in diagnosis of diphtheria. Proc. Soc. Exp. Biol. and Med., 1955, 88:368.
- Sharpe, E.: A serological classification of Lactobacilli. J. Gen. Microbiol., 1955, 12:107.
- Tittsler, R. P., Pederson, C. S., Snell, E. E., Hedlin, D., and Niven, C. F.: Symposium on the lactic acid bacteria. Bact. Rev., 1952, 16:227.

## Spherical Bacteria

SPHERICAL, or nearly spherical, bacteria are found in several divisions of the class Schizomycetes, such as the genus of soil autotrophs, *Nitrosococcus*; the genera *Thiosarcina* and *Thioplyococcus*, of the suborder Rhodobacteriineae; and the genus *Myxococcus* of the order Myxobacteriales. In this chapter we shall confine our discussion to the larger and more important groups: Micrococcaceae, Neisseriaceae and Streptococceae. These are all heterotrophic, non-sporeforming, and non-motile. All are gram-positive except the Neisseriaceae.

These three groups are readily differentiated, and subdivided into genera, as follows:

1. **Family Micrococcaceae:** (divide in more than 1 plane) (Fig. 36-1, A, C, D)
  - (a) Divide in 2 planes at 90°: *Gaffkya*\* (square groups)
  - (b) Divide in 3 planes at 90°: *Sarcina*† (cubical packets)†
  - (c) Divide in 3 planes, at various angles: *Micrococcus* (irregular groups like bunches of grapes)
2. **Tribe Streptococceae:** (divide in 1 plane only) (Fig. 36-1, B)
  - (a) Cling together in long chains after fission: *Streptococcus*
  - (b) Tend to separate into short chains or pairs: *Diplococcus*
  - (c) Chains of cells like *Streptococcus* but typically form very large, polysaccharide capsules or zoogical masses in vegetable juices (sugar vats, sauerkraut, etc.): *Leuconostoc*
3. **Family Neisseriaceae:** (gram-negative; morphologically distinctive, flattened, coffeebean-shaped cocci; divide in 1 plane only) (Fig. 36-6)
  - (a) Remain as pairs with distinctive flattened appearance: *Neisseria*
  - (b) Irregular groups; strictly anaerobic: *Veillonella* (not discussed here).

### 1. THE FAMILY MICROCOCCACEAE

**General Characters.** The organisms of the family Micrococcaceae are usually nearly spherical in form. Three genera are included, differentiated

\* Gaffky was a famous bacteriologist who first described these organisms.

† *Sarcina* is from a Latin word meaning packet.

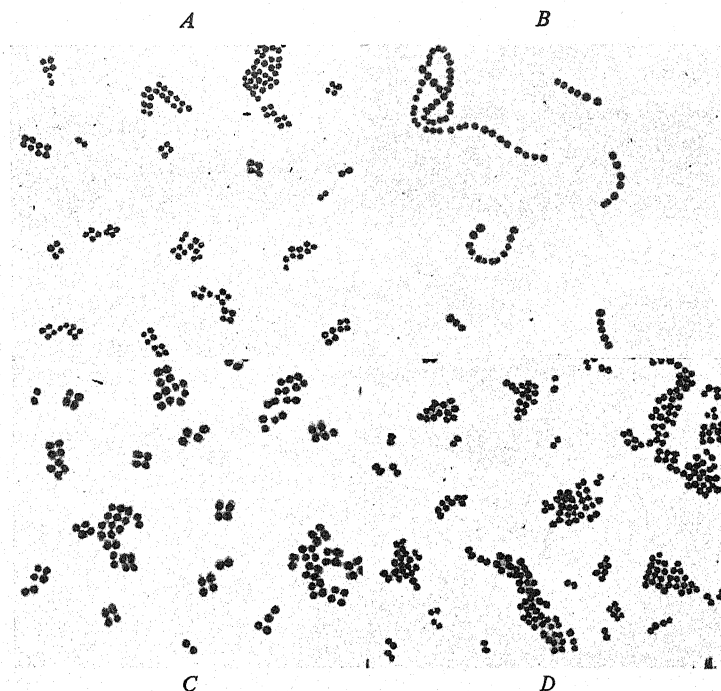


Fig. 36-1. Various forms of Micrococcaceae. A, *Gaffkya*; B, *Streptococcus*; C, *Sarcina*; D, *Micrococcus* (staphylococcus). ( $\times 1000$ .) (Ford, Textbook of Bacteriology, W. B. Saunders Co.)

primarily by the arrangement of the cocci. Their principal differential characters have been listed above.

The family includes some pathogenic species, notably *Micrococcus pyogenes* (var. *aureus*), *Micrococcus pyogenes* (var. *albus*) and *Gaffkya tetragena*. Some of these (especially *M. pyogenes*) cause serious diseases of man and animals. However, most of the Micrococcaceae are saprophytes.

All of the Micrococcaceae grow well facultatively at temperatures of from  $30^{\circ}$  to  $40^{\circ}$  C on simple organic media such as extract agar or peptone solution. A large majority form opaque, butyrous colonies colored with white or yellow pigments; various shades of red and orange are especially common among the saprophytic types. Micrococci have a marked tolerance for NaCl and can be isolated from mixed cultures on media containing 5 to 8 per cent salt, a concentration which inhibits growth of many other organisms.

**Micrococcaceae in Nature.** Except for *Micrococcus pyogenes* and *Gaffkya tetragena*, which are pathogenic, the Micrococcaceae are of importance mainly as scavengers. They are widely distributed in milk, soil, air, dust and water, especially where putrefaction and decay are in progress. They are among the common air and dust-borne contaminants in laboratory cultures. Many digest proteins like gelatin and casein and attack various carbohydrates and other organic substances. Several species (*M. cereus*, *M. perflavus*, *M. caseolyticus*) are of some commercial importance in the ripening and flavoring of cheese since they attack casein and lactose with the production of aromatic

substances having pleasing flavors. Some produce various undesirable conditions such as "ropy milk" (*M. viscosus* and *M. cremoriviscosi*).

### PATHOGENIC MICROCOCCI

Because the name *Staphylococcus* is one of long standing and common usage, it will be continued here in a non-specific sense to mean pathogenic micrococci. Staphylococci have the general properties of other micrococci.

The staphylococci are usually to be found on the skin or mucous membranes of the animal body, especially of the nose and mouth, where they often occur in large numbers even under "normal" conditions. The two principal species are: (1) *Micrococcus pyogenes* (var. *aureus*), distinguished by its golden-yellow pigment and notorious as the cause of suppurative (pus-forming) conditions: mastitis of cows, boils, carbuncles, and internal abscesses in man; (2) *M. pyogenes* (var. *albus*).

The most pathogenic staphylococci generally: (1) ferment mannite; (2) liquefy gelatin; (3) produce coagulase (an enzyme-like principle which causes oxalated, heparinized or citrated blood plasma to clot); (4) produce golden yellow pigment; and (5) produce lipase. However, these properties are neither constant nor exclusive among staphylococci, and it is sometimes difficult to make a distinction between staphylococci and the non-pathogenic micrococci. True staphylococci, especially *M. pyogenes* var. *aureus*, often produce hemolytic colonies when cultivated on the surface of blood agar. However, many non-pathogenic species of micrococci and other bacteria, including various rod forms, are also hemolytic. In addition, staphylococci produce leukocidins and several toxins which destroy certain tissue cells.

**ENTEROTOXIN.** Staphylococci are also notorious as a cause of a very common type of food poisoning. This is because many strains of staphylococci excrete a heat-stable poison called *enterotoxin*.\* When ingested, staphylococcal enterotoxin causes nausea, vomiting, diarrhea and prostration. It is more fully discussed in Chapter 43.

**Gaffkya** are generally regarded as saprophytic (with few exceptions) so far as man is concerned. However, this species is very pathogenic for mice and some other forms of life. It is also pathogenic for lobsters, producing a commercially costly disease with a high fatality rate. The diseased lobsters have a pink discoloration on the ventral side of the abdomen; the blood, normally blue, is pink and watery. The organisms are found in large numbers in the blood and tissues and Koch's postulates have been demonstrated with them. The tetrads of *Gaffkya* in the lesions are characteristically heavily encapsulated.

### TRIBE STREPTOCOCCEAE

A primary subdivision of streptococci may be made into three large groups: lactic, enterococcus, and pyogenic. Many of these produce characteristic appearances around their colonies in blood agar in Petri plates. Since these appearances are constantly referred to in discussing streptococci, they are described here, along with the procedure for making blood-agar plates.

**Blood-agar Plates.** To determine the blood-agar type a tube containing about 15 ml of melted, meat-infusion agar, cooled to about 45° C (still fluid,

\* A toxin causing enteritis or diarrhea. Do not confuse with endotoxin.



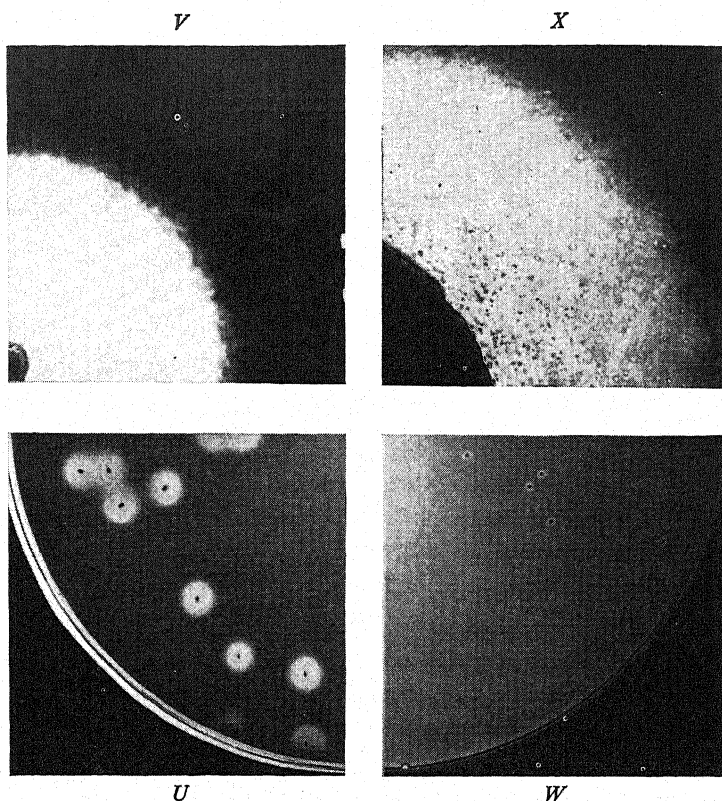


Fig. 36-2. Colonies of hemolytic streptococci in blood agar. *U*, clear zones of complete hemolysis around colonies of beta type (*Strep. pyogenes*, Lancefield group A), natural size. *V*, one beta-type colony enlarged to show edge of colony at lower left and absence of erythrocytes in clear hemolyzed zone. *W*, small hemolytic zones of alpha-type colony, *Strep. mitis*, natural size. *X*, one alpha-type colony enlarged to show edge of colony at lower left, with many intact erythrocytes in hemolyzed zone. (Preparations by Dr. Elaine L. Updyke. Photo courtesy of Communicable Disease Center, U. S. Public Health Service, Atlanta, Ga.)

yet not hot enough to injure the microorganisms), is inoculated with a loopful of pus, milk, broth culture, or other material containing the desired streptococci. About 5 per cent sterile blood is added aseptically and well mixed with the agar. The mixture is poured into plates and incubated for twenty-four hours; at about 20° C for lactic streptococci and 37° C for others.

#### BLOOD-AGAR TYPES

Four main types of streptococci are recognized on the basis of their action in blood-agar plates: the alpha and beta hemolytic types, the double-zone beta hemolytic type, and the gamma type.

**Alpha-type Hemolytic Streptococci.** These colonies are surrounded by a zone of hemolysis and also a zone of discolored erythrocytes *close in* around the *deep* colonies. These erythrocytes have a green or brownish-green color. Peripheral to this inner ring of discolored cells the outer zone of clear

hemolysis may be of great or small extent, and may sometimes be so small as to coincide with the zone of green cells. It usually widens on refrigeration of the plate (Fig. 36-2, *W, X*).

Only the use of a microscope can be relied upon to make the distinction and *only colonies which are deep in the agar* are always thus characterized, surface colonies sometimes producing deceptive appearances. *Pathogenic* green-producing varieties (alpha type) of hemolytic streptococci are often called "streptococcus viridans."

**Beta-Type Hemolytic Streptococci.** The hemolytic zones of streptococci of this type in blood-agar plates are seen to be entirely clear and free from any intact erythrocytes (Fig. 36-2, *U, V*). Such streptococci are loosely spoken of under the general term of "streptococcus hemolyticus" or "hemolytic strep."

**Double-Zone Beta-Type Streptococci.** Certain species, almost exclusively of bovine origin, and not uncommon in dairy products, after producing a zone of hemolysis like that of other beta-type streptococci, on standing at room temperature or on refrigeration produce a second ring of hemolysis separated from the first by a ring of red erythrocytes (Fig. 36-3).

**Gamma Type.** When colonies of streptococci in blood-agar plates show no visible change in the blood cells surrounding the colony they are said to be of the gamma or indifferent or non-hemolytic type.

#### 1. THE LACTIC STREPTOCOCCI

This group contains *Streptococcus lactis*,\* the common, milk-souring streptococcus, useful in the manufacturing dairy industry. *S. lactis* are usually of the gamma type in blood agar but may produce green colonies. *S. lactis* is always present in market milk, even of the best quality. It occurs in cow dung, dust, soil, on plants, in utensils, and so on and its entrance into the milk is easily explained. Its survival in such environments shows that it is a relatively

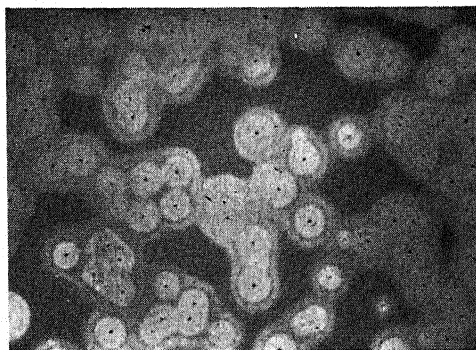


Fig. 36-3. Double zone beta-type streptococcus colonies in blood agar; incubated at 37° C and then held at low temperature several hours; natural size. Note the usual clear zone of complete hemolysis, surrounded by a zone of unhemolyzed cells, then by an outer zone of clear hemolysis. (Preparation by Dr. Elaine L. Updyke.) (Photo courtesy of Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.)

\* *S. lactis* probably represents a group of variants or closely similar species, rather than a single, well-defined species.

hardy organism. It is quite harmless to man. Its vital role in the dairy and food industries is discussed in Chapters 42 and 43.

It sometimes forms long chains but occurs chiefly in short chains or pairs and the cells tend toward an oval shape. It grows rapidly, causing souring of milk at summer temperatures; usually overgrowing and suppressing the development of other organisms, some of which might otherwise cause the milk to putrefy. *S. lactis* can readily be cultivated in sterile milk, or on agar containing milk or whey or tomato juice, at about 25° C. It grows best in the presence of glucose or lactose.

**Streptococci Related to *S. Lactis*.** There are numerous closely related streptococci, all of them forming lactic acid. One of these is *S. cremoris*. It tends to form long chains. Pure cultures of this organism, mixed with *S. lactis* and some species of *Leuconostoc* (which are very similar), are used in making butter and cheese. The object is to ensure that the milk or cream is soured by organisms (*S. cremoris*) which yield a pleasant, "buttery" flavor. Some other varieties of lactic streptococci are carefully avoided since they cause undesirable flavors, or conditions of sliminess ("ropy milk").

## 2. THE ENTEROCOCCI

Streptococci of this group inhabit the intestine of man and animals, dung, sewage, etc. Many of the enterococci have a tendency to produce short chains and pairs of plump, ovoid cocci, and are commonly found in clumps suggestive of micrococci. They were first described as micrococci.

The group is characterized by wide tolerance of heat and cold and other influences unfavorable to other streptococci: 6.5 per cent NaCl; bile (low surface tension); the presence of 0.1 per cent methylene blue; pH 9.6; and conditions of life in feces (see Table 20). Enterococci are even more hardy than the lactic group. The enterococci are most commonly represented by *S. faecalis*. Colonies of this organism usually produce alpha-type zones of hemolysis in blood-agar plates. Some species of enterococci produce beta-type colonies and some produce gamma-type. The group is seen, therefore, to be somewhat heterogeneous.

Related to *S. faecalis* are *S. liquefaciens* and *S. zymogenes*, the last differing from other enterococci in producing beta-type zones in blood agar. Both species occur commonly in dung and dairy products. They grow readily on the organic media commonly used in the laboratory. Unlike *S. lactis* they grow better at about 35° C than at 25° C.

*S. liquefaciens* and *S. zymogenes* hydrolyze proteins. As a result, they give rise to strong bitter flavors in cheese. They have been found occasionally in certain pathological conditions.

## 3. THE PYOGENIC STREPTOCOCCI

The pyogenic (pus-producing) streptococci may be subdivided into: (a) the beta-hemolytic, pyogenic streptococci, represented by *S. pyogenes*; and (b) the alpha-hemolytic or "viridans" pyogenic streptococci, represented by *S. mitis*.

(a) **The Beta Hemolytic species** are very different from the lactic and enterococcic groups. In general they are relatively fragile organisms, adapted to a parasitic life in and on the mammalian body. Generally they are studied in



medical and diagnostic laboratories. They are best cultivated at 37° C in media containing serum or blood. Some of them are among the most deadly pathogens. There are several serological groups of these streptococci as follows.

**LANCEFIELD GROUPS OF BETA-TYPE STREPTOCOCCI.** Lancefield made extracts of massive cultures of different strains of these bacteria by means of hot, N/20 HCl. These extracts contain specific, somatic, antigen (carbohydrate or C substances). The extracts are used as antigens in precipitin tests.

By means of such precipitin tests several distinct groups of beta-type hemolytic streptococci can be differentiated with respect to origin. Lancefield designated these groups by letters: A, B, C, D, E, F, G, etc., according to source or other characters. They are accordingly known as *Lancefield groups* (Remember, these apply distinctly to *beta-type* streptococci.)\*

**GROUP A.** Biological properties distinguishing group A streptococci from other groups are shown in Table 20. Group A streptococci include those causing scarlet fever, septic sore throat, empyema, puerperal sepsis, and many other serious, epidemic, and acute pyogenic diseases in human beings. These are represented by the type species of the genus, *S. pyogenes*.

**VARIETIES AND TYPES OF GROUP A.** Two colony forms of streptococci of group A have been discerned: a smooth, regular, glossy form often called the T form, and a dull, slightly-irregular form called *matte* or M. Each M variant contains a different protein, somatic antigen. These evoke type-specific precipitins. Over 40 serological types ("M types") of group A streptococci have been found.

The importance of determining serological (Lancefield) group and M type of beta hemolytic streptococci becomes clear when it is realized that resistance to Group A streptococcal infection is type-specific. For example, one may have resistance to a type 6 streptococcus, yet succumb in a type 19 epidemic with the same disease. The importance of typing in the study of methods by which streptococcal disease is spread can scarcely be overestimated. For example, the whole problem of the control of rheumatic heart disease appears bound up with the epidemiology of streptococcal infection.

**GROUP B STREPTOCOCCI** differ from all others in hydrolyzing sodium hippurate and in producing double zones of beta hemolysis (Fig. 36-3).

Hemolytic streptococci of group B are usually of bovine origin and harmless for man, but are occasionally found in human infections. *S. agalactiae*, an important member of group B, is of particular interest to the farmer because it causes severe mastitis in cattle and stoppage of milk flow.

**GROUP C STREPTOCOCCI.** This group is of a dual nature with respect to origin, containing (1) strains of human origin culturally indistinguishable from group A and which cause many similar conditions; and (2) strains from various animal sources. The latter rarely occur in human infections.

**GROUP D STREPTOCOCCI.** These are members of the group of enterococci (*S. zymogenes*) which are discussed elsewhere.

**GROUP G STREPTOCOCCI.** These strains are related to group C strains, and closely resemble group A strains in important biochemical properties. They

\* A few alpha-type streptococci and some other organisms have been found to contain some of these antigens.

are generally not highly pathogenic, but vary in this respect. They are frequent in human infections.

GROUPS E, F, H, ETC. These groups contain such small numbers of strains that they will not be discussed in detail. They have been found occasionally in animals and human beings, and also in dairy products.

(b) **The Alpha Hemolytic ("Viridans") Streptococci.** These may be thought of as comprising three groups. One consists of delicate, fragile, highly-adapted, parasitic, pyogenic streptococci. These are found mainly in the mammalian oral and respiratory tracts and are capable of producing serious infections. They are represented by *S. mitis*, of man, *S. equinus* of horses, and *S. bovis* of cattle.

A second group is represented by *S. salivarius*, a species common in the human mouth, hardier than *S. mitis*, and generally saprophytic. *S. salivarius* is distinguished by forming thick, gummy colonies if cultivated on media containing about 5 per cent sucrose. The gumminess is due to the synthesis of levulans from the sucrose and is a good example of the tendency of many bacterial species to synthesize polysaccharides. Another common species of saprophytic, viridans streptococci is *S. thermophilus*. It is distinctive in growing well at temperatures up to about 65° C. This organism gives much trouble to commercial milk pasteurizers because it is not destroyed by pasteurization, surviving 30 minutes at 65° C. It can grow to large numbers in pasteurizing vats, and appears in the milk upon examination by the health departments. It casts unjustifiable doubt on the efficacy of the pasteurization process. However, it can be eliminated by good sanitization with hot water. It is not pathogenic. It occurs in dung and barn dust.

A third group of alpha type, hemolytic, streptococci is made up of species of enterococci. These are commonly represented by *S. faecalis*, which has been discussed with the enterococci. These are often pyogenic.

**PATHOGENESIS BY ALPHA-TYPE STREPTOCOCCI.** Although constantly present in the normal mouth, usually without deleterious effects, these organisms can at times gain a foothold in the body. They tend to cause chronic, but none the less dangerous, suppurative diseases. They are frequently found in abscessed teeth, sinuses, and diseased tonsils and are always present in saliva. From teeth and tonsils they may be carried to the joints and produce rheumatic conditions. They can also infect the heart valves causing a serious heart disease, *bacterial endocarditis*. *S. faecalis* is frequently involved.

#### GENUS DIPLOCOCCUS

The principal species in this genus is *Diplococcus pneumoniae*, cause of lobar pneumonia, a frequent cause of death before the advent of chemotherapy.

British writers logically refer to *Diplococcus pneumoniae* as *Streptococcus pneumoniae* since the organisms have the major characteristics of streptococci. They often form chains, usually made up of from two to eight pairs of cocci. They are encapsulated. The pneumococci are rarely spherical, having the form of short artillery projectiles placed base to base. They are gram-positive. In blood-agar plates they produce alpha-type zones. They are facultative, heterotrophic and parasitic. Methods of cultivation and study are like those used for pyogenic streptococci. They are found in the saliva and sputum of

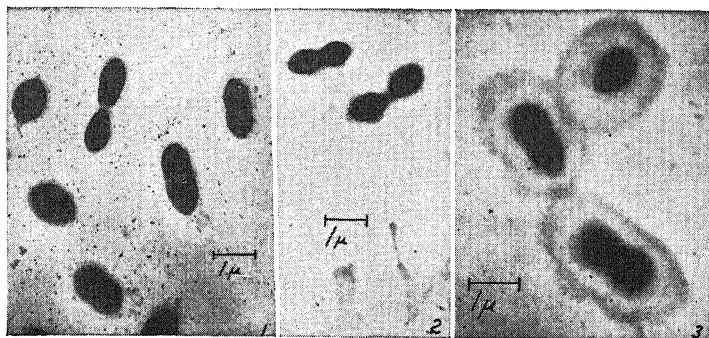


Fig. 36-4. Electronographs of *Diplococcus pneumoniae*. The first picture shows the capsules in their normal state. The center picture shows the capsules virtually unaffected by serum of a heterologous type. The picture on the right shows the effect of homologous type serum on the capsules—a well-marked quellung reaction. (From Mudd, Heinmets, and Anderson, in *The J. Exp. Med.*, vol. 78.)

patients with lobar pneumonia and, like streptococci, also occur frequently in the normal mouth and throat.

Pneumococci are extremely *pathogenic for white mice* when freshly isolated from the body. Advantage is often taken of this fact to isolate pneumococci from sputum of patients for diagnosis. The sputum is injected into the mice intraperitoneally. After six to twenty-four hours the mice die or become very ill, and enormous numbers of pneumococci are found in the peritoneal cavity and heart blood. The cocci found on the peritoneum of the mouse may be identified by: (1) their morphology; (2) their *capsules* (Fig. 36-4); (3) their *solubility* in bile or in solutions of various surface-tension reducers, of which bile is one; (4) ability to ferment inulin (see Table 20).

**Serological Types of Pneumococci.** Pneumococci may be divided into more than seventy serological types which are designated by Roman numerals. These are analogous to the Lancefield groups of streptococci. Serological type-specificity of pneumococci is conferred by their capsules. Each type has its own, specific, capsular, carbohydrate antigen. Immune sera are available representing each type.

**QUELLUNG REACTION.** If encapsulated pneumococci are mixed with a *type-specific* immune serum swelling of the capsules is seen (Fig. 36-4). This is spoken of as a quellung (German for *swelling*) reaction. It was first described by Neufeld and is sometimes called the Neufeld reaction.

The quellung method of serological typing or grouping is applicable to the study of many other species of encapsulated organisms, not only of medical but also of agricultural importance. If deprived of their capsules, as when they vary toward the rough phase, such organisms are: (1) immunologically indistinguishable; and (2) (in the case of pathogens) avirulent.

These facts relating to the occurrence of specific carbohydrate substances in or on cells of bacteria are of much wider than medical interest because they illustrate a phenomenon of fundamental importance which is encountered frequently in microbiology—the occurrence within a species, of immunological “types” or groups which differ because of the chemical differences in their carbohydrates.



**TRANSFORMATION OF PNEUMOCOCCAL TYPES.** An interesting agent capable of inducing genetically stable alteration in type specificity of pneumococci is a deoxyribonucleic acid fraction extracted chemically from certain pneumococci. This has been described in Chapters 5 and 15.

**Pathogenic Action of Pneumococci.** Pneumococci are highly invasive organisms and, like beta hemolytic streptococci, can infect many different parts of the body and, according to their localization cause meningitis, septicemia, peritonitis, empyema, sinusitis, etc. The name pneumococcus is, of course, derived from the fact that they were first seen as the cause of pneumonia.

*The method of transmission of pneumonia* is chiefly through droplets of infected saliva and nasal and pulmonary mucus, and by inhalation of infected dust. Kissing undoubtedly transmits the infection as well as other respiratory pathogens but obviously not every such infection results in disease. Romance has a powerful ally in natural resistance to infectious disease. Pneumococci survive desiccation very well, and thus are found in dust in patients' rooms, hospital wards, etc.

#### GENUS LEUCONOSTOC

The name of this streptococcus-like genus is derived from the name of a blue-green alga, *Nostoc*, and the Greek *leukos*, meaning colorless. *Nostoc*, the alga, is characterized by spherical cells occurring in tangled chains with a thick, gelatinous, outer coating. *Leuconostoc*, the bacteria, resemble *Nostoc* in forming spherical cells in tangled chains. At least two species (*L. mesenteroides* and *L. dextranicum*), when growing in carbohydrate-rich media,

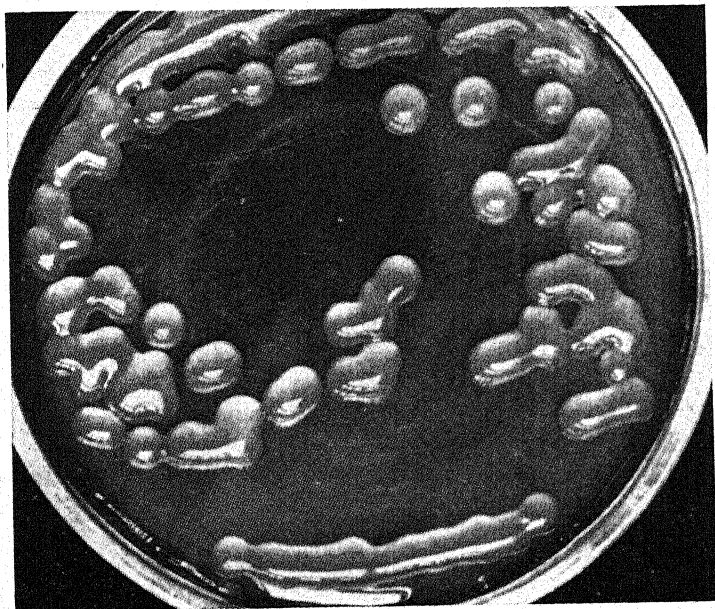


Fig. 36-5. *Leuconostoc mesenteroides*. Three-day culture on 10 per cent raw sugar agar, incubated at room temperature. (McCleskey, C. S., Faville, L. W., and Barnett, R. O., in *J. Bact.*, vol. 54.)



synthesize thick, masses of gummy polysaccharide (dextran) about themselves (Fig. 36-5). (Compare with *Strep. salivarius*.)

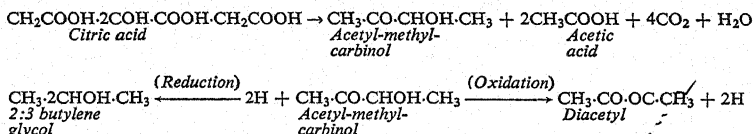
A detailed study of *L. mesenteroides* and *L. dextranicum* has shown that there are various types, differentiated serologically and by the amount and molecular nature of gum formed, as well as by amount of CO<sub>2</sub> produced, etc.

*Leuconostoc* are widely distributed on growing plants. They ferment actively and produce acid in such carbohydrate-rich plant materials as sauerkraut, ensilage, and in plant juices such as are used in making cane sugar, beet sugar, etc. They constitute a great nuisance in the sugar refining industry, clogging vats, pipes and machines. Indeed, so much of the specific carbohydrate of *Leuconostoc* is left in commercial cane, beet, and other sugars that specific precipitin tests can be obtained by mixing a little "sugar water" with suitable immune serum.

***Leuconostoc citrovorum*.** This organism, when not growing in sugar-rich solutions, produces little or none of the gum which is so characteristic of the genus. It then so closely resembles *S. lactis* that some have regarded it as a streptococcus (*S. citrovorum*). (It has also been classified, probably most logically, as a species of *Pediococcus*.) A very closely related species is called *L. paracitrovorum*. These organisms are closely allied to *S. lactis* and are commonly found in its company and have some of its principal characters.

**DIACETYL.** The important point about *L. citrovorum* and its allies is the fact that they decompose citric acid with the formation of acetic acid, CO<sub>2</sub> and, most important, acetyl-methyl-carbinol and diacetyl. The latter two compounds are responsible for the pleasant, buttery flavor of dairy products. Consequently, pure cultures of *L. citrovorum*, etc., are commonly added to milk or cream that is to be made into butter, cheese and the like. Citric acid is also often added to increase the amount of diacetyl formed.

It is important to note that diacetyl is formed by the *oxidation* of acetyl-methyl-carbinol in an *acid* medium:



Sometimes a little sulfuric, lactic or other harmless acid is added to ensure proper pH. Also, aeration is necessary or, as shown in the foregoing reaction, the acetyl methyl carbinol, instead of being *oxidized* to diacetyl, is *reduced* to 2:3 butylene glycol which is tasteless (see above reactions). These facts are of vital importance to manufacturing dairymen.

An important branch of the dairy industry is the selection, propagation, maintenance and sale of pure cultures of *S. lactis*, *L. citrovorum*, various lactobacilli, and mixtures of these for the manufacture of butter, fermented milks, yoghurt, cheeses, etc. (see Chapter 44).

## THE LACTIC BACTERIA

### RECAPITULATION

We might recapitulate the main facts concerning the lactic organisms in the following manner:

## I. Produce lactic acid in significant amounts from lactose and glucose.

A. Rod-forms: *Lactobacillus*

## 1. Mainly in dairy products (Homofermentative)

*L. lactis**L. acidophilus**L. caucasicus**L. casei**L. thermophilus*

## 2. Mainly in plant products (sauerkraut, pickles, ensilage, etc.)

## a. Homofermentative

*L. plantarum*

## b. Heterofermentative

*L. brevis**L. fermenti* } CO<sub>2</sub> formed

## B. Coccus forms: Streptococceae

## 1. Mainly in dairy products

## a. Homofermentative

*S. lactis**S. cremoris* } (important as lactic acid producers)*S. thermophilus* (resists pasteurization)*S. liquefaciens* (proteolytic) (bitter flavors in cheese)

## b. Heterofermentative

*Leuconostoc citrovorum* } Diacetyl-buttery flavor*L. dextranicum*

## 2. Mainly in plant products

*Leuconostoc mesenteroides* (slimy growths)

## THE FAMILY NEISSERIACEAE

The family Neisseriaceae includes two genera, *Neisseria* and *Veillonella*. The latter are small, gram-negative, anaerobic cocci which occur chiefly in the normal mouth. *Veillonella* has been relatively little studied and will not be discussed in detail.

## GENUS NEISSERIA

This genus derives its name from a German bacteriologist, Neisser, who, in 1879, discovered and studied one of the two most important species. The *Neisseria* are rather small, gram-negative diplococci, each cell characteristically flattened where it is in contact with its mate, each cell having somewhat the shape of a coffee bean (Fig. 36-6). The various species of *Neisseria* (about eleven) are indistinguishable morphologically. They are aerobic and grow best at 35° to 37° C. Unlike the ubiquitous and busy lactic organisms, the *Neisseria* are found only in or on the human body.

Of the several species of *Neisseria*, two commonly cause disease. *N. gonorrhoeae* (the gonococcus) causes gonorrhea, while *N. meningitidis* (the meningococcus) causes epidemic meningitis. *N. gonorrhoeae* and *N. meningitidis* are very much alike and one is probably a minor variant of the other. They are classical examples of microorganisms that have become highly adapted to an existence of parasitism on man.

In moist, warm material as pus, urine, serum, or cultures in incubators,

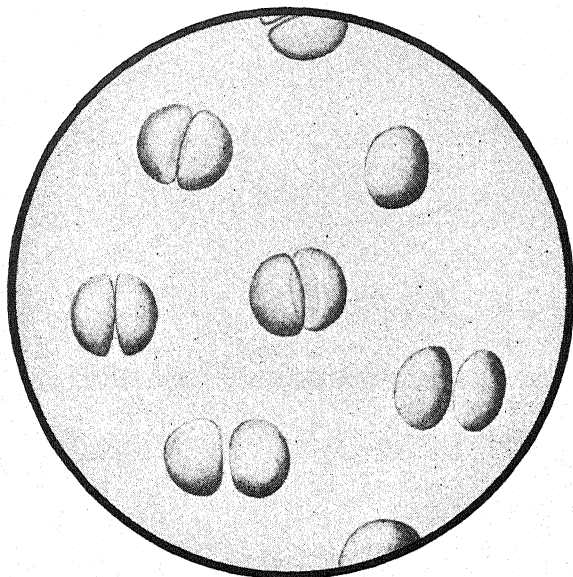


Fig. 36-6. Diagrammatic enlargement of *Neisseria gonorrhoeae*. The magnification is in the range of the electron microscope. Note the rounded, hemispherical appearance of each cell of the pairs.

the meningococci and gonococci autolyze and die rather rapidly. They do not survive for long periods outside the body as do typhoid, tubercle and diphtheria bacilli or streptococci. Drying is very deleterious to gonococci; chilling to meningococci. They grow best at 35° to 37° C, on infusion media containing blood heated to 90° C ("chocolate agar"), in a very humid atmosphere, with 5 to 10 per cent CO<sub>2</sub>. The colonies are from 1 to 4 mm in diameter, clear, colorless, moist and fragile.

These cultural characteristics serve to differentiate the meningococci and gonococci from all other species of *Neisseria*: *N. flava*, *N. sicca*, etc. These others grow well on blood-free media at temperatures as low as 25° C and are moderately resistant to drying, chilling and light. These species, while parasites in a broad sense, being restricted to a life on a mammalian host, are usually quite harmless. However, under certain conditions some of them may cause meningitis, and some can cause vaginal infections in little girls which are sometimes confused with gonorrhea with tragic results. Therefore, no diagnosis of gonorrhea can be said to be complete and accurate without a full bacteriological study of the organism involved. Some institutional outbreaks of vulvovaginitis in little girls are due entirely to infections by *N. catarrhalis* or *N. sicca*.

Except for the differences noted above the *Neisseria* are all very similar. *N. meningitidis* and *N. gonorrhoeae* are especially closely related to each other, being distinguished with difficulty even by serological means and fermentation tests. Indeed, *N. gonorrhoeae* has been found to cause meningitis while *N. meningitidis* has been isolated from conditions clinically indistinguishable from gonorrhea.

The meningococci are separable into four main serological groups on the basis of agglutination reactions. These are groups I, II, IIa, and IV. A quellung reaction for grouping is available, analogous to that used in typing pneumococci, since freshly isolated strains of meningococci possess capsules.

**The Oxidase Test.** All of the *Neisseria* produce an enzyme (oxidase) which causes a 1 per cent solution of dimethyl-paraphenylene diamine to turn, successively, pink, rose, magenta, and finally black. The oxidase test is applied by moistening a colony of the suspected organism with a drop or two of the dye solution. The changes in color begin a in few moments. The same test for oxidase can be made on all sorts of microorganisms besides *Neisseria* and is a valuable differential method for general use.

**The Catalase Test.** The test for catalase is made by putting a drop or two of  $H_2O_2$  on any "suspected" colony. If catalase is present, bubbles of oxygen will appear almost instantly. The catalase test is widely used in microbiology. All *Neisseria* produce catalase. So do many other aerobic organisms.

Cultures from the genitalia rarely contain more than one type of *Neisseria*. If more than one type are found, all may be of diagnostic significance.

#### *Pathogenic Action of the Neisseria*

**Gonorrhea.** This is one of several diseases commonly spoken of as "venereal diseases," deriving this appellation from the name of Venus, goddess of love. The inappropriateness of this term will become obvious in the discussion of the infections.

Gonorrhea is an inflammatory disease due to infection, by *N. gonorrhoeae*, of the mucous surfaces and adjacent glandular structures of the reproductive organs of men and women. Much pus forms, and appears as a white discharge (leukorrhea) from the genitalia. It is an alarmingly prevalent disease, hundreds of thousands of cases being under medical care in the United States in 1956. There are undoubtedly many other, probably a million or more, unreported and untreated cases.

Infection of the genitalia with *N. gonorrhoeae* occurs virtually exclusively through sexual intercourse. Gonorrhea is seldom fatal but is sometimes difficult to cure. Patients often believe themselves cured only to find, later, that the disease has reappeared in a chronic form. The sulfonamide drugs promised for a time to eliminate gonorrhea, but drug-fast strains of gonococci rapidly developed and indiscriminate use of the drugs by the medically ignorant has robbed such therapy of most of its effectiveness. Penicillin now offers the best hope for cure in all cases. It can be readily understood that careless, ignorant or malicious people can spread gonorrhea widely. Prostitution is one of the chief means by which the disease is propagated. Adequate treatment with penicillin very close to the time of exposure will prevent many cases from developing. Inadequate treatment is, in several respects, worse than none.

Gonorrhea, untreated, often results in sterility. The intense inflammation caused by the endotoxins of the organism (possibly capsular substance) destroys the lining tissues of the genito-urinary tract, the tissue being replaced with scars. Such scars obstruct the *fallopian tubes* of the female and the *vas deferens* of the male (tubes through which the reproductive cells pass). Such scarred obstructions are called *strictures*. Stricture of the urethra in the male

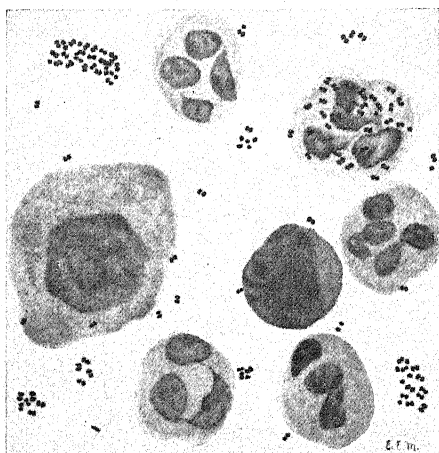


Fig. 36-7. Gonococcus from urethral pus, stained with methylene-blue ( $\times 1000$ ). (Ford, Textbook of Bacteriology.)

also interferes with urination and may require surgical intervention. Gonococci sometimes invade the body, localizing in the joints and the heart valves. In the former case, a very painful and stubborn type of arthritis results while in the latter case a very damaging disease of the heart occurs, with permanent injury and sometimes death.

**Gonorrheal Ophthalmia.** An intensely painful inflammatory infection of the eye (*ophthalmia*) results when gonococci are rubbed into the eye. Loss of sight usually results in a few days, unless treatment is prompt. A gonorrheal mother may infect her child's eyes at birth. Due to this fact most cities, states and countries require that physicians, nurses or midwives attending births, *regardless of any circumstances*, instill into the eyes of the infant a few drops of weak (1%) silver nitrate, penicillin, or other appropriate disinfectant solution. This rapidly destroys gonococci before they can start an infection in the eye. Approved disinfectants are obtainable at any health department or drug store, ready for use.

**LABORATORY DIAGNOSIS OF GONORRHEA.** The diagnosis of *acute* gonorrheal infection in the adult *male* is usually based on microscopic examination of the pus stained by Gram's method. The gonococci appear *within* the leukocytes (Fig. 36-7). Such organisms in adult males with acute urethritis are rarely any but gonococci. In the female genito-urinary tract many other organisms are present and, as noted above, respiratory *Neisseria* may cause infection in little girls, being transmitted by hands, towels, etc., soiled with oral or nasal secretions. Isolation and complete cultural and serological identification of the organism is therefore of importance.

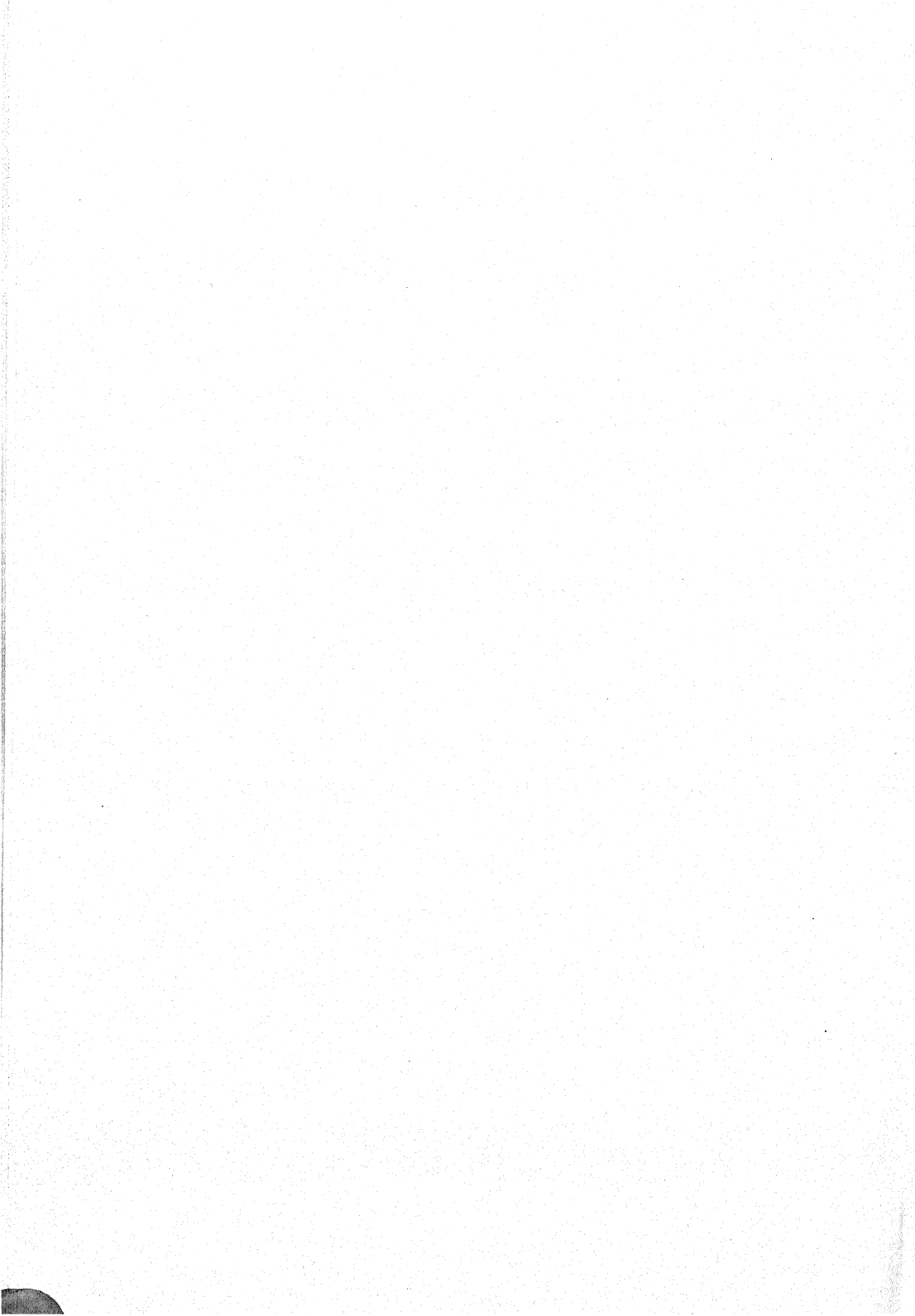
**Meningitis.** The term meningitis is drawn from pathology and means, simply, inflammation of the membranes (meninges) covering the brain and spinal cord. It may be due to mechanical irritations, viruses, or many kinds of bacteria, both pathogens and saprophytes, which may localize in the meninges. The meningococcus is the only common cause of *epidemics* of meningitis. The organisms are transmitted as are other respiratory microorganisms, in oral and nasal secretions.

CARRIERS of meningococci are common, but *meningitis* is not. There is evi-

dence that the meningococcus often causes conditions like rhinitis, "catarrh" or purulent "colds" which heal and attract no particular attention because the etiological agent is unsuspected.

## REFERENCES

- Branham, S. E.: Serological relationships among meningococci. *Bact. Rev.*, 1953, 17:175.
- Branham, S. E.: Milestones in the history of the meningococcus. *Canadian J. Micr.*, 1956, 2:175.
- Dack, G. M.: Food poisoning. *Ann. Rev. Microbiol.*, 1953, 7:327.
- Editorial: Albert Neisser and the gonococcus. *Am. J. Pub. Health*, 1955, 45:95.
- Heffron, Roderick: *Pneumonia*. Commonwealth Fund, New York, 1939.
- Larkin, E. P., Litsky, W., and Fuller, J. E.: Fecal streptococci in frozen foods, I and II. *Appl. Micr.*, 1955, 3:98, 102.
- Parrino, P. S., O'Shaughnessy, E. J., and White, J. D.: Standardization of diagnostic methods for gonococcal infections. *Am. J. Pub. Health*, 1955, 45:457.
- Ravenholt, R. T., and LaVeck, G. D.: Staphylococcal disease—an obstetric, pediatric and community problem. *Am. J. Pub. Health*, 1956, 46:1287.
- Rogers, D. E., and others: Staphylococcal infections. *Ann. N. Y. Acad. Sci.*, 1956, 65 (Art. 3):57 et seq.
- Scherp, H. W.: *Neisseria and neisserial infections*. *Ann. Rev. Microbiol.*, 1955, 9:319.
- The Child with Rheumatic Fever: Children's Bureau Folder No. 42—1955. U. S. Gov't. Printing Office, Washington 25, D. C.
- White, B.: *The Biology of Pneumococcus*. Commonwealth Fund, New York, 1938.

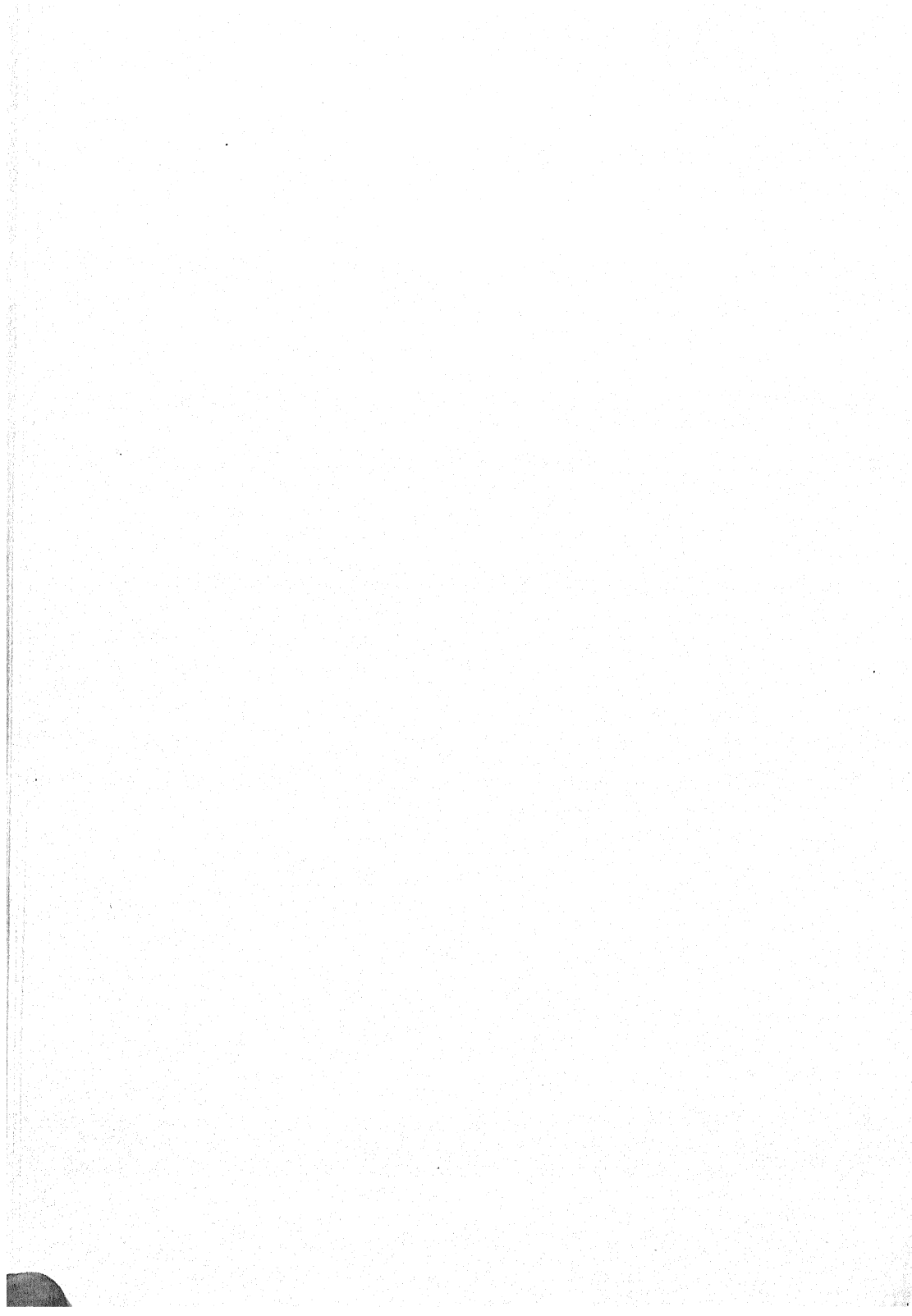


## SECTION 5

# *The Microbiology of Special Environments*

HAVING LEARNED in sections 1 to 4 a good deal about microorganisms in general and about bacteria in particular we turn, in this final section, to environments in which microorganisms are found, and to the activities of the microorganisms in those environments. We find, for the most part, familiar names—organisms previously described as laboratory subjects—now seen “at home” as it were, in their natural habitats. We see how and where they live, what they do there, and how their activities affect man. We travel from the bottoms of the most profound marine chasms to “the desert and illimitable air” over the arctic oceans; from the warm, fertile furrow to the icy, rushing mountain torrent; from nauseous volcanic hot springs to the whiskey distillery or dairy bucket. Everywhere we find microorganisms at their appointed tasks. They were working at some of their secret objectives long before human beings appeared on this planet. It seems probable that they will continue in their mysterious round long after the disappearance of man; and wholly oblivious of him.





## The Microbiology of Water

### CONTROLLING FACTORS

THE MICROSCOPIC flora of water depends to a great extent on: (1) *Nutrient substances* in the water e.g., ferrous iron for "iron bacteria;"  $H_2S$  for "sulfur bacteria;" decaying vegetation or sewage for heterotrophic bacteria; and so on. (2) *Substances unfavorable* to certain organisms. For example, sea water is too saline for many species, while  $H_2S$ , produced by many putrefactive species, is unfavorable to several other species. Many organisms produce antibiotic substances. Acids, as  $H_2SO_4$  from *Thiobacillus*, or organic acids, alcohols, etc., resulting from fermentation in soils or stagnant ooze, or toxic metals like copper from industrial wastes, are unfavorable to many organisms. (3) The bacterial flora of water depends partly on the presence and numbers of *other living organisms*, especially protozoa and bacteriophages, both of which destroy billions of bacteria. (4) *Physical factors* such as temperature, amount of dissolved oxygen, exposure to sunlight (for photosynthetic species) and, as indicated above, pH. (5) *The sources* from which organisms are contributed.

### AQUEOUS ENVIRONMENTS

For convenience, we may divide natural bodies of water into (1) *fresh waters* (rivers, lakes, ground waters, etc.); (2) *sea waters* (oceans and landward extensions); (3) *saline and hot waters* (Dead Sea, Great Salt Lake, thermal springs). Each type of environment has its own distinctive flora which cannot thrive in other environments (the *indigenous flora*). There are also numerous species which can grow equally well in more than one situation. The physiological adaptations of some of the more restricted species are quite curious and interesting.

1. **Bacteria in Fresh Waters.** Much depends on the location and nature of the body of fresh water; on whether it is a rapid stream high in snow-covered mountains, a meandering lowland river in a thickly populated, agricultural area, or a stagnant pool in a woodland swamp. In any case, part of the flora of any surface water is introduced periodically by rain wash from adjacent land surface; some by dust from the air, some by continuous growth of indigenous organisms.

**UNPOLLUTED WATERS.** In lakes and rivers free from sewage pollution the concentration of nutrients in solution is usually much lower than in polluted

streams like the Hudson, Danube or Ganges rivers. Consider a placid woodland pool, fed by surface runoff and springs. The water is clear and looks "pure." Here food is found in concentration only near the shores, bottom, and at solid surfaces (see Chapter 13).

The numbers of bacteria floating free in the water away from such zones are quite limited; often only a dozen or so per ml. These may include various species of soil saprophytes which can grow to some extent in the small amount of organic and mineral substances in solution in the water: species of *Micrococcus*, *Flavobacterium*, *Achromobacterium*, *Bacillus*, *Proteus*, *Leptospira* and others. If there is much decaying organic matter at the bottom, species of *Clostridium* and other anaerobes, strict and facultative, are often found, including sulfur bacteria. Caulobacteriales and Chlamydobacteriales may be found growing on the surfaces of rocks and logs near the shore. If  $H_2S$  is being produced by decomposition at the bottom and if the pool is not too shaded, species of photosynthetic bacteria may be present.

If, during a windstorm, a large tree falls into the water and stirs up the bottom sediment, especially if it is during a warm spell in the summer, the whole flora changes almost momentarily. Cellulose-digesters and fermentative types thrive. The organic matter stirred up from the bottom furnishes a rich and varied pabulum. Numerous species of saprophytes, previously present in small numbers, multiply enormously and some, previously numerous, are suppressed by newly multiplying, antagonistic species. Total numbers per ml may rise to 100,000 or more until an equilibrium is again reached.

In a high mountain stream derived from melting snow the numbers and variety of microorganisms to be found are ordinarily small. Unless the stream runs over polluted soil, or soil rich in decaying vegetable matter, the water is likely to be almost sterile. It may contain a few spores of *Bacillus*, molds or yeasts, but they will not be very actively germinating because of the low temperature. They have probably been caught from the air by the falling snow. A few other microorganisms: micrococci, corynebacteria, gram-negative rods mostly from dust of the air or from soil, caught by snow or rain, might be found. They would not be multiplying much, due to cold and lack of dissolved nutrient substances.

**POLLUTED WATERS.** The lower Hudson River has a flora representative of sewage pollution. One may assume that *Escherichia coli*\* and other Enterobacteriaceae\* as well as enterococci† and various species of intestinal *Clostridium* are present in large numbers. Many soil saprophytes like *Spirillum*, *Vibrio*, *Sarcina*, *Micrococcus*, *Mycobacterium*, *Bacillus*, yeasts, molds, *Micromonospora*, *Leptospira* and other spirochetes, *Beggiatoa*, *Sphaerotilus*, and many others would also find the organic, fecal matter good pabulum.

In the mud and ooze at the bottom, the O-R potential is low and anaerobic species (*Clostridium*, *Desulfovibrio* and various facultative bacteria, their nature depending on the physicochemical nature of the sediment) exist.

In the more aerated, surface layers strict anaerobes do not thrive, and the odors and tastes of putrefaction and fermentation are not found. The total numbers of microorganisms may reach into the millions per ml of water.

\* Gram-negative, facultative, non-sporeforming rods, which are usually found in the intestinal tract of man or animals.

† Intestinal streptococci.

There is nothing constant or necessarily predictable about the flora of such a body of water except within wide limits. Conditions in such a tidal river change hourly and the flora changes in response to conditions. The temporary pollution from large ships like the "Constitution" or "Queen Elizabeth" is a case in point.

In any body of water, saprophytic organisms serve the purpose of scavengers. They decompose organic wastes and make them available as food for other creatures in the water: higher plants, protozoa, worms, etc. These in turn support fish and other commercially useful marine or aquatic life, and so contribute to human welfare. The suppression of saprophytic microorganisms and other aquatic life by excessive sewage pollution and by microbicidal industrial waste is one of the major problems of the progress of civilization.

### THE PSEUDOMONADACEAE AND ENTEROBACTERIACEAE

In populous areas organisms found in fresh surface waters are often of intestinal origin. Most of these belong to the family of Enterobacteriaceae and the group of enterococci. Many of these same species also live in soil and water as saprophytes. It will be helpful at this point to consider the Enterobacteriaceae in detail and to compare them with several similar and related organisms of the family Pseudomonadaceae, many of which are also common in soil and water, sewage, etc.

#### FAMILY PSEUDOMONADACEAE\*

The family Pseudomonadaceae is an extensive one, comprising some 150 (recognized) species of the genus *Pseudomonas*; 47 of *Xanthomonas*; about a dozen of *Methanomonas*, *Acetobacter*, *Protaminobacter* and *Mycoplana*; and about 45 species of curved or spiral forms like *Vibrio* and *Spirillum*.

**General Properties.** Except for some species of *Spirillum*, all are gram-negative; non-sporeforming; chemosynthetic; not sheathed, stalked, or sulfur-storing; unbranching (with the possible exception of *Mycoplana*). All but a few questionable species are motile by one or more polar flagella.

This large group need not concern us unduly. We have already devoted a chapter to the spiral and curved forms. They are common aquatic bacteria. We may by-pass the *Methanomonas*,† *Protaminobacter*† and *Mycoplana*† at this time because, while important scavengers, they are small groups, of interest mainly because of their curious, specialized metabolic characters. *Acetobacter* we shall meet later (Chapter 44) as a wealthy vinegar manufacturer. *Xanthomonas* (while very much like *Pseudomonas*, and often washed into water from soil and plants) we shall dismiss at this point because these organisms are more conveniently studied as the cause of "rots" and other diseases of plants. This leaves us to consider in detail only the genus *Pseudomonas*.

\* The Pseudomonadaceae are characterized by polar flagella. Their family name is derived from the Greek word *pseudo*, meaning similar to, and the word *monas*, which is the name of a group of protozoa having polar flagella.

† *Methanomonas*: autotrophic; oxidizes  $\text{CH}_4$  (derived from cellulose decomposition) as a source of energy; common in swamps. *Protaminobacter*: can decompose alkylamines; common in soil and water. *Mycoplana*: cells said to branch slightly; can metabolize phenol and related compounds as source of energy; common in soil.

GENUS *PSEUDOMONAS*

These are among the most common and widely distributed bacteria. They are easily cultivated on any ordinary, organic, laboratory media at temperatures of from 15° to 40° C (best at 25° C) at pH from about 6.5 to 8.0 (best around 7.2). They are enzymically active, hydrolyzing a wide variety of proteins, fats, carbohydrates and other organic compounds. Thus they are excellent and ubiquitous scavengers. They are principally aerobic; some are facultative. They are found in soil, fresh waters and ocean waters and decomposing organic matter, including sewage. Only two representative species need be detailed at this point.

*Pseudomonas fluorescens* produces a greenish-yellow, fluorescent pigment in cultures. There are numerous similar species, widely distributed.

*Pseudomonas aeruginosa* is type-species of the genus. In addition to the yellow-green pigment, characteristic of many *Pseudomonas*, it produces a turquoise-blue pigment, *pyocyanin*, which may be extracted from broth culture with chloroform.

***Pseudomonas* as Pathogen.** *Ps. aeruginosa*, a common saprophyte, is not infrequently found in wounds or ulcers which have not healed promptly. Some outbreaks of diarrhea in adults and especially among newborn children are said to be caused by this organism. *Ps. aeruginosa* is also able to cause a leaf-rot disease in tobacco and lettuce. *Ps. aeruginosa* causes a fatal disease in poultry.

## FAMILY ENTEROBACTERIACEAE

The family Enterobacteriaceae includes numerous species which are commonly found in the intestinal tract of man and/or animals. Hence, the name of the family. The family comprises about ten genera of closely related bacteria subdivided into hundreds of species and serological types. Some workers divide the family into four main groups as shown in Table 21.

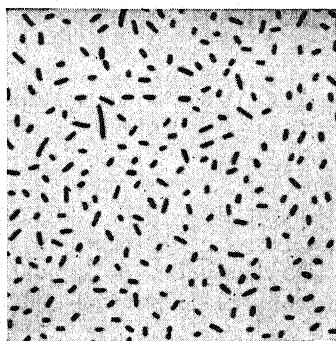
Table 21. *The Enterobacteriaceae.*

PIGMENT NOT PRODUCED		PIGMENT PRODUCED
Lactose fermented in 24 hours, with gas	<i>Escherichia</i> (usually no perceptible capsules) <i>Aerobacter</i> } probably identical; (usually <i>Klebsiella</i> } heavy capsules)	<i>Serratia</i> (blood red pigment on starch media at 22° C)
Lactose fermented only after 48 hours or longer	<i>Paracolobactrum</i> (gas; not proteolytic) <i>Erwinia</i> (mostly no gas; mostly proteolytic)	
Lactose not fermented	<i>Salmonella</i> (motile) } (do not decompose <i>Shigella</i> (non-motile) } urea; not proteolytic) <i>Proteus</i> (motile; decomposes urea; proteolytic)	

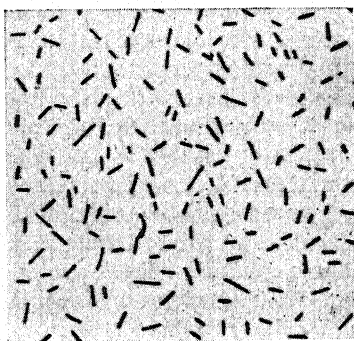
**General Properties.** The Enterobacteriaceae are like the non-spiral *Pseudomonas* in a number of ways (Fig. 37-1). Their principal distinguishing characters are shown in comparison with Pseudomonadaceae in Table 22.

**Table 22.** Comparison of Non-Spiral *Pseudomonadaceae* and *Enterobacteriaceae*.

CHARACTERISTICS	PSEUDOMONADACEAE	ENTEROBACTERIACEAE
1. Morphology	Simple, straight rods	Same as <i>Pseudomonadaceae</i>
2. Staining	Gram-negative	" " "
3. Spores	None	" " "
4. Motility	All motile	Species vary
5. Flagellation	Polar	Peritrichous
6. Growth requirements	Heterotrophs (complex); pH 6 to 8; optimum 15 to 25° C	Heterotrophs (simple); require organic C; pH 6 to 8; optimum 25 to 30° C
7. Habitat	Outer environment: soil, water, organic matter	Intestinal tract; outer environment: soil, water, organic matter
8. Enzymatic activities	Proteolytic; somewhat active fermenters	Fermentative; a few are proteolytic
9. Reduction of NaNO <sub>3</sub>	Species vary	Active
10. Relation to oxygen	Preferentially aerobic (a few exceptions)	Facultative
11. Pigments	Yellowish, fluorescent; pyocyanin (blue)	None (except <i>Serratia</i> , blood red).



A



B

Fig. 37-1. A, *Proteus vulgaris*, typical *Enterobacteriaceae*, plain agar, twenty-four hours, stained with crystal violet. B, *Pseudomonas fluorescens*. The morphological differences between the typical *Enterobacteriaceae* and *Pseudomonadaceae* shown here are neither constant nor distinctive. The labels could be interchanged and still be correct. (Ford, Textbook of Bacteriology.)

**Enteric Genera.** The most distinctively enteric organisms of this family are included in seven genera: *Proteus*, *Escherichia*, *Aerobacter*, *Klebsiella*, *Paracolobactrum*, *Salmonella* and *Shigella*. At least four genera cause disease and are detailed in Chapter 39.

#### GENUS PROTEUS

This genus consists of soil and water saprophytes, common in decaying animal or vegetable matter and often found in the human intestine or as opportunists in infections (especially cystitis\*) of man and causing diseases in lower animals. These are also situations in which some species of *Pseudomonas* and Enterobacteriaceae are found. *Proteus* species occur in polluted water and, while not highly active in fermentation, they readily decompose proteins and thus are valuable scavengers. The power to *hydrolyze urea* is a *distinctive character* of *Proteus*, which is used in diagnosis of enteric disease (see *Salmonella*). Cultures of *Proteus* usually have a disagreeable, foetid odor.

**Proteus Colonies.** *Proteus* colonies are distinctive in that they usually spread rapidly over the surface of solid media, forming a thin, grey, almost transparent film which may escape notice entirely unless it is especially sought. This form of growth is often designated as the *H form*, or "swarming." An aflagellate, non-motile variant form of *Proteus* occurs (called the *O form*) of which the colonies are small, discrete and circular. These peculiarities are of great importance in medical diagnostic work.

#### THE COLIFORM GROUP

**Genus Escherichia.**† This genus comprises several species of special interest to the sanitarian since the organisms occur commonly in the intestinal tract of man and animals. *Escherichia coli* is adapted to a constant life in the intestines. While found in sewage and in recently-feces-polluted materials it is not well adapted to continuous life in the outer world.

*E. freundii* and *E. intermedium* are very similar species but commonly live in the outer world as well as in the intestinal tract. Consequently, their presence may or may not indicate fecal or sewage pollution. As between the distinctly intestinal *E. coli* and the largely outer-environmental forms such as *Proteus*, *Aerobacter* or *Klebsiella* (to be discussed), these two species are often spoken of as "intermediates." All of these species are so similar that they are often spoken of together as the "coliform group."

**ESCHERICHIA COLI.** This species has all of the general properties of the family Enterobacteriaceae. It is distinguished from *all of the other coliforms* by its *inability* to utilize citrate in place of glucose in an otherwise wholly inorganic medium; a reflection, perhaps, of its adaptation to a sub-parasitic existence. Some varieties of *E. coli*, indeed, are genuine parasites and cause infections of the intestinal and urinary tract.

Distinctive properties of *E. coli* and other organisms discussed in this chapter are detailed in Table 23.

**Genera Aerobacter and Klebsiella.** These organisms are much like the

\* Infectious inflammation of the urinary bladder.

† Escherich was a famous German bacteriologist, pupil of Koch.

other coliforms but are more commonly soil and water organisms than intestinal. However, they can live in either environment.

The only species of *Aerobacter* is *A. cloacae*. Closely similar to it is *Klebsiella aerogenes*.\* They differ mainly in that *A. cloacae* is proteolytic and motile, while *K. aerogenes* is not proteolytic and usually non-motile.

**Table 23.** *Distinctive Properties of the Coliform Organisms.\**

SPECIES	LACTOSE	DEX-TROSE	SUCROSE	I	M	V	C	GELATIN	MOTILITY	GAS RATIO
<i>Esch. coli</i>	⊕	⊕	⊕	+	+	-	-	-	+	H:CO <sub>2</sub> > 1
<i>Esch. freundii</i>	⊕	⊕	⊕	-	+	-	+	-	+	H:CO <sub>2</sub> > 1
<i>Klebs. aerogenes</i>	⊕	⊕	⊕	-	-	+	+	-	-	H:CO <sub>2</sub> < 1
<i>Aero. cloacae</i>	⊕	⊕	⊕	-	-	+	+	+	+	H:CO <sub>2</sub> < 1

\* + = positive test.

- = negative test.

⊕ = acid and gas formed.

#### SANITARY RELATIONSHIPS OF THE COLIFORM GROUP

Every well-informed person knows that *Salmonella typhi* (the typhoid bacillus) is transmitted by food, milk, or water supplies which have become polluted with the urine or feces of a person harboring this organism. In thickly populated districts practically every body of surface water receives some fecal pollution due to discharge of sewage or due to the careless habits of ignorant people with respect to urination and defecation. Sometimes people may be perfectly healthy yet harbor typhoid bacilli in their intestinal or urinary tract and discharge them in large numbers, thus infecting water supplies, milk, food and other substances which they handle. Such persons are called "carriers" of typhoid. Other pathogenic Enterobacteriaceae (*Salmonella*, *Shigella*, etc., Chapter 39) as well as viruses of poliomyelitis, hepatitis, and other pathogenic microorganisms, may accompany *S. typhi* and be transmitted in identical fashion.

**Pollution of water with fecal material**, whether infected or not, is obviously undesirable, both from the standpoint of danger of infection and for purely esthetic reasons. The detection of fecal bacteria in food or domestic water is, therefore, of importance in determining its suitability for human consumption. For every typhoid bacillus or other pathogen in polluted water supplies there are usually millions of coliform organisms.

**Indices of Fecal Pollution.** Because of their common occurrence in feces, the coliforms serve, by their presence in water, food, etc., as an *index of fecal pollution*. Methods for their detection and enumeration in water and foods

\* The organism formerly called *Aerobacter aerogenes* is now discussed as *Klebsiella aerogenes*.



are carefully prescribed by the American Public Health Association, and are used daily in every health-department laboratory. A series of steps, called *Presumptive Test*, *Confirmed Test* and *Completed Test*, are carried out systematically. These are shown in Chart 1.

Two other groups of bacteria invariably present in human (and animal) feces are *enterococci* (especially *S. faecalis*) and *Clostridium* (especially *Cl. perfringens*). These two species are easily isolated from water by the use of relatively simple methods of selective cultivation and are readily identified. They, also, are used frequently, especially in British practice, as indices of fecal pollution, both in waters and foods.

**SIGNIFICANCE OF INDEX ORGANISMS.** The survival time of these three indicator organisms in water is of significance. The enterococci seem unable to multiply significantly in open water and do not survive long. Their presence in considerable numbers, therefore, suggests relatively recent pollution; a few hours or days. The *coliforms*, especially *E. freundii*, *E. intermedium*, *Aerobacter* and *Klebsiella*, generally outnumber the streptococci and, being often able to multiply to some extent in open, polluted waters, may survive for weeks or months, depending on conditions in the water. *Cl. perfringens*, because of its resistant spores, can survive indefinitely. The presence of this organism to the exclusion of the others, therefore, suggests pollution a considerable time ago, if it occurred at all. Clostridia grow naturally in many soils and waters.

None of these organisms is a *perfect* index of human pollution because all occur in the soil or in animal dung, or both, as well as in human excrement. Examination of any water or food for these index species *must be supplemented* by a sanitary survey-examination of the terrain or situation to evaluate the probability of human fecal pollution before final conclusions or legal condemnation.

**The "Membrane" or "Millipore" Filter (M.F.) Method.** Some of the difficulties in determining the presence of coliform organisms by the methods just outlined lie in the facts that: (a) only relatively small quantities of water can be examined at one time; (b) several days are required for the incubation of the successive cultures; (c) the test is largely qualitative, only relatively rough estimates of numbers of coliform organisms being feasible; (d) it requires considerable amounts of expensive medium and equipment; (e) it is not readily done in the field "on the spot," necessitating transportation of samples to the distant laboratory and consequently very undesirable changes in coliform content of the samples.

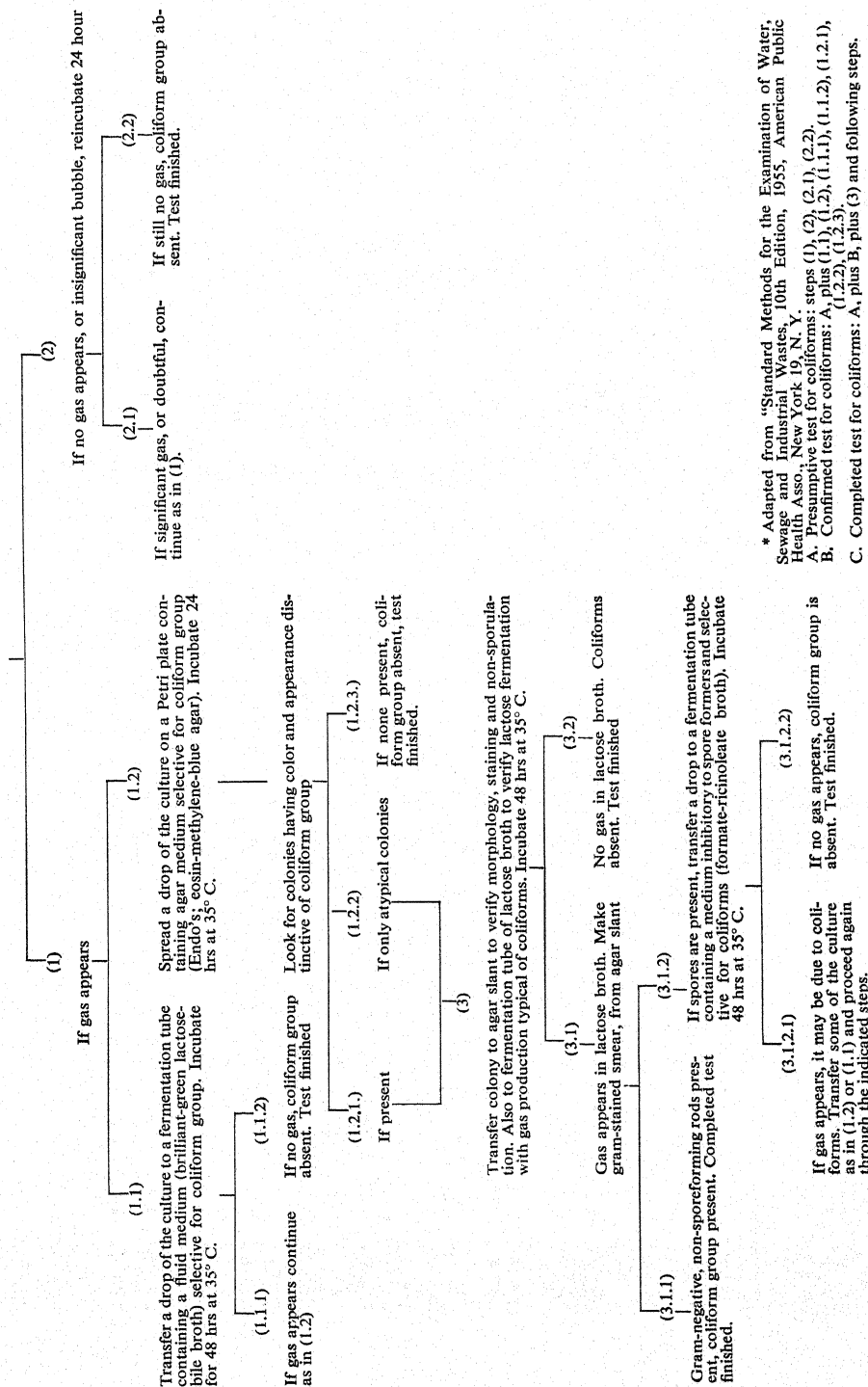
By means of "membrane" or "Millipore" filters (see Chapter 13) it is possible to filter, rapidly, large samples of water to be tested for coliform or, indeed, any and all organisms. The bacteria in the sample are held on the surface of the filter membrane (Fig. 37-2). By methods already indicated it is possible, within 20 hours at 35° C not only to enumerate the coliform colonies by their distinctive color but to finish the procedures involved in the *Completed test*, in the Standard A.P.H.A. procedure. (See Chart 1.) Compact field kits for use on the spot are available (Fig. 37-3).

A difficulty is that the filter membrane cannot be used with waters containing any considerable amount of silt or sediment likely to clog the filter. The Standard Test and the M.F. method do not measure exactly the same

Chart 1.

Outline of Official Completed Test for Coliform Organisms in Fluids.\*

Inoculate measured samples into fermentation tubes containing tryptose broth and an agent (sodium lauryl sulfate) inhibitory to non-coliforms. Incubate 24 ( $\pm$  2) hrs at 35° C ( $\pm$  0.5°).



\* Adapted from "Standard Methods for the Examination of Water, Sewage and Industrial Wastes, 10th Edition, 1955, American Public Health Assn., New York 19, N. Y.

A. Presumptive test for coliforms: steps (1), (2), (2.1), (2.2).

B. Confirmed test for coliforms: A, plus (1.1), (1.2), (1.1.1), (1.1.2), (1.2.1), (1.2.2), (1.2.3).

C. Completed test for coliforms: A, plus B, plus (3) and following steps.

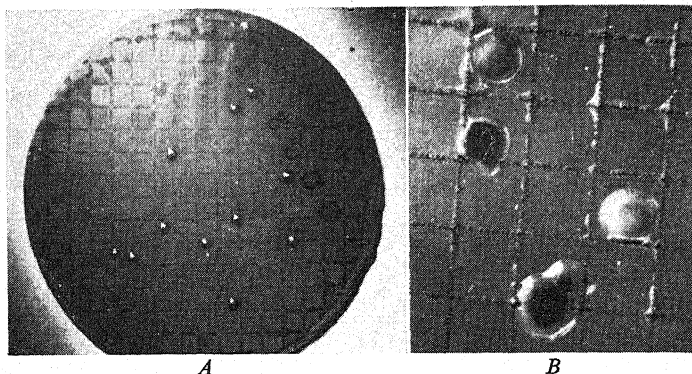


Fig. 37-2. *A*, membrane or ultrafilter disk with colonies of *Escherichia coli*. The disk is on a circular pad saturated with Endo medium, which is selective for coliform organisms. *Esch. coli* colonies are seen here as dark and glistening (about actual size). They are actually deep magenta in color and have a green-violet metallic iridescence. *B*, section of membrane filter disk with colonies of coliform organisms. *Escherichia coli* colonies appear here as whitish, opaque, and slightly mounded. *Klebsiella aerogenes* are larger, more mucoid, with dark centers (magnified  $\times 4$ ). (Photos courtesy of Environmental Health Center, U. S. Public Health Service, Cincinnati, Ohio.)

flora and neither gives results which, on the basis of present knowledge, may be expressed accurately in terms of the other.

#### DIFFERENTIATION OF FECAL AND NON-FECAL TYPES

For many purposes of water and food sanitation it is sufficient to know whether or not *any* coliform organisms are present. However, it is sometimes considered desirable to distinguish between the more definitely intestinal *Esch. coli* and the other coliforms. Depending on the situation, these may, or may not, represent fecal pollution.

The procedures are technically not difficult. Having obtained satisfactory pure cultures from either the Standard or M.F. test, they are differentiated on the basis of indol production, the methyl red test, the Voges-Proskauer reaction and ability to utilize sodium citrate as a sole source of carbon (Table 23). The technical details may be found in "Standard Methods for the Examination of Water, Sewage and Industrial Wastes" 1955, American Public Health Association.

**Imvic Formula.** A mnemonic\* "IMViC" is used, with plus and minus signs, to express the differences between the organisms in a "formula." *I* stands for indol reaction, *M* for methyl red reaction, *V* for the acetyl-methyl-carbinol test (originated by Voges-Proskauer), *i* is for euphony, and *C* for growth in mineral solution containing citrate as a sole source of carbon.

Thus, an organism of the coliform group designated as "IMViC ++ --" would be *Esch. coli* since this gives positive indol and methyl red reactions but negative Voges-Proskauer and citrate reactions. These symbols are used in Table 23.

\* A memory-aiding device.

## GENUS PARACOLOBACTRUM

This group resembles *Aerobacter*, *Klebsiella*, and *Escherichia* in all major respects, but differs in *very slow* fermentation of lactose. These slow-lactose fermenters, often called "*paracolons*," have long confused sanitary and medical microbiologists because they are often mistaken for the pathogenic *Salmonella* (Chapter 39), which does not ferment lactose at all. Some groups of paracolons can readily be differentiated from *Salmonella* by the use of media containing 0.0075 per cent KCN. Most *Salmonella* do not grow in this medium while many paracolons do. Some species of paracolons are pathogens, causing enteritis and diarrhea.

## MARINE BACTERIA

Marine bacteria have been defined as those which will not grow on media without sea water. For general studies of marine bacteria a recommended medium contains: sea water (aged) 1000 ml; peptone, 5.0 gm;  $\text{FePO}_4$ , 0.1 gm; agar, 15 gm.

**Marine Zones.** The sea has been divided into *biotic zones*: the *benthic zone* or sea floor (benthos); the *pelagic*, usually thought of as the upper layers; the *oceanic* or open sea; the *neritic* or shallow (200-meter) or coastal waters; the *littoral* or close-in, beach or tidal inland waters; a *euphotic* or lighted zone; and an *aphotic* or dark zone. The largest numbers and varieties of microorganisms are found in the shallow waters near land (neritic or littoral). Food is more plentiful here.

**MARINE ENVIRONMENTS.** TEMPERATURES range from  $40^\circ\text{C}$  in some littoral waters of the tropics to  $-2^\circ\text{C}$  in polar regions (sea water freezes at about  $-2^\circ\text{C}$ ).



Fig. 37-3. Field monitoring kit.

**SALINITY.** Most truly marine bacteria are sensitive to small changes in salinity above or below that of sea water; i.e., they are *stenohaline*. Some bacteria peculiar to salt lakes (Dead Sea, etc.) *require* salinities of 13% or more (*halophilic*).

**PRESSURES** at the floor of the profoundest depths become very high. At one mile the hydrostatic pressure is around 2000 lbs. per square inch. Yet many bacteria thrive at six miles and deeper where the temperatures range around  $-2^{\circ}\text{C}$ . They seem to be adapted to these conditions. For example, in the laboratory, many grow only at  $2.5^{\circ}\text{C}$  and 15,000 lbs. pressure per square inch. Those requiring high pressures are said to be *barophilic*.

It is curious that in samples of mud from the bottom of the sea off California, at depths of about 5000 feet where the temperature ranges constantly below  $10^{\circ}\text{C}$ , obligate thermophils, growing *only* at temperatures of  $60^{\circ}\text{C}$  or over, were found.

**Photogenic Bacteria.** There are several species of photogenic bacteria, indigenous to the sea. They may be cultivated upon sea-water agar with peptone. *Pseudomonas phosphorescens*, isolated from luminous marine fish, is one common example.

**Marine Bacteria and Petroleum.** Very interesting observations have been made concerning the possible role of marine microorganisms in the formation of petroleum. Marine bacteria are enzymatically active, like soil and sewage bacteria. The various organisms inhabiting deep ocean beds and marine sediments can, as a group, attack almost any sort of organic matter. There is some evidence, although it is not conclusive, that these marine anaerobic microorganisms can transform certain organic substances into petroleum-like matter.

#### REFERENCES

- Allen, C. H., and Fabian, F. W.: Comparison of *E. coli* and *S. faecalis* as test organisms to determine the sanitary quality of food. J. Milk and Food Technol., 1954, 17:234, 237.
- Anonymous. Water pollution in the United States. Pub. Health Serv. Publ. No. 64. Gov't. Printing Off., Washington 25, D. C., 1951.
- Bush, J. H.: The membrane filter applied to water supply control. Water and Sewage Works, 1955, 102:436.
- Cooper, M. L., Walters, E. W., and Keller, H. M.: Detection of a new serotype of *Escherichia coli* 0127: B8, associated with acute diarrhea in infants. J. Bact., 1955, 69:689.
- Design, Equipment and Operation of Swimming Pools and Other Public Bathing Places. American Public Health Assoc. Report, New York, 1955.
- Editorial: Infectious agents in sewage. J.A.M.A., 1956, 160:877.
- Editorial: Pathogenicity of *Escherichia coli*. J.A.M.A., 1954, 154:837.
- Edwards, P. R., and Ewing, W. H.: Identification of Enterobacteriaceae. Burgess Publ. Co., Minneapolis 15, Minn., 1955.
- Edwards, P. R., and Fife, M. A.: Studies on the Klebsiella-Aerobacter group of bacteria. J. Bact., 1955, 70:382.
- Edwards, P. R., and Fife, M. A.: Cyanide media in the differentiation of enteric bacteria. Appl. Micr., 1956, 4:46.
- Eliassen, R.: A realistic appraisal of membrane filters for water quality control. Water and Sewage Works, 1955, 102:523.
- Eveland, W. C., and Faber, J. E.: Antigenic studies of a group of paracolon bacteria (32011 group). J. Inf. Dis., 1953, 93:226.
- Ewing, W. H., and Edwards, P. R.: Isolation and preliminary identification of *Escherichia coli* serotypes associated with cases of diarrhea of the newborn. Pub. Health Lab., 1954, 12:75.

- Ewing, W. H., Tanner, K. E., and Dennard, D. A.: The providence group: an intermediate group of enteric bacteria. *J. Inf. Dis.*, 1954, 94:134.
- Greenberg, A. E., Yee, L., and Hartmann, F. W.: The present state of knowledge concerning the membrane filter in water bacteriology. *The Pub. Health Lab.*, 1956, 14:73.
- Heukelekian, H.: Microbiology of water and sewage. *Ann. Rev. Microbiol.*, 1953, 7:461.
- Heyl, J. G.: On the occurrence and the significance of paracolon bacteria in The Netherlands. A. V. Leeuwenhoek *J. Micr. and Serol.*, 1954, 20:406.
- Kelly, S., Winsor, J., and Winkelstein, W.: Poliomyelitis and other enteric viruses in sewage. *Am. J. Pub. Health*, 1957, 47:72.
- Lieber, M.: A critique on the membrane filter. *Water and Sewage Works*, 1955, 102:400.
- Litsky, W., Mallman, W. L., and Fifield, C. W.: Comparison of the most probable numbers of *Escherichia coli* and enterococci in river waters. *Am. J. Pub. Health*, 1955, 45:1049.
- MacLeod, R. A., Onofrey, E., and Norris, M. E.: Nutrition and metabolism of marine bacteria. *J. Bact.*, 1954, 68:680.
- McBee, R. H., and McBee, V. H.: The incidence of thermophilic bacteria in arctic soils and waters. *J. Bact.*, 1956, 71:182.
- Morris, W., and Weaver, R. H.: Streptococci as indices of pollution in well-waters. *App. Micr.*, 1954, 2:282.
- Odum, E. P.: *Fundamentals of Ecology*. W. B. Saunders Co., Philadelphia, 1953.
- Pederson, H. T., Jr., and Skinner, C. E.: A comparison of standard lactose broth with lauryl sulfate broth and with the Eijkman method for demonstrating fecal coliform bacteria. *Appl. Micr.*, 1955, 3:55.
- Ringen, L. M., and Drake, C. H.: A study of the incidence of *Pseudomonas aeruginosa* from various natural sources. *J. Bact.*, 1952, 64:841.
- Shipe, E. L., and Fields, A.: Coliform determinations from swimming and wading pools by membrane filtration and the most probable number method. *Pub. Health Lab.*, 1955, 13:44.
- Slanetz, L. W., and Bartley, C. H.: Evaluation of membrane filters for the determination of numbers of coliform bacteria in water. *Appl. Micr.*, 1955, 3:46.
- Standard Methods for the Examination of Water, Sewage and Industrial Wastes. 10th ed. American Public Health Association, New York, 1955.
- Ware, G. C.: Bacteriological examination of polluted water and sewage effluents. *Lab. Pract.*, (London), 1955, 4:444.
- West, M. G., and Edwards, P. R.: The Bethesda-Ballerup Group of Paracolon Bacteria. *Pub. Health Monogr. No. 22*, 1954. U. S. Gov't Printing Office, Washington 25, D. C.
- ZoBell, C. E.: *Marine Microbiology*. Chronica Botanica Co., Waltham, Mass., 1946.

## Sanitation of Drinking Water and Sewage Disposal

INFECTIOUS DISEASES often transmitted by unsanitized drinking water are *typhoid fever* (salmonellosis), (Chapter 39); Asiatic cholera (Chapter 32); *amebic* (protozoan) *dysentery*; *bacillary dysentery* (shigellosis), (Chapter 39); *leptospirosis*, (Chapter 31); *viral hepatitis* (catarrhal jaundice), (Chapter 45); tularemia “rabbit fever,” (Chapter 39); infections due to certain “tape worms” (trematodes) may also be transmitted by water. Of these various diseases those commonly transmitted by water in the United States are printed above in *italics*. The others are only occasionally water-borne or occur only in the tropics or the Orient.

Bodies of water are convenient places for the disposal of sewage and other refuse and it was not uncommon, until even as late as the latter part of the nineteenth century, for a city to pour its sewage into a body of water at one point and build the intake for a drinking-water supply not far away. Consequently, sewage got into the drinking water and epidemics of intestinal infection occurred.

Springs, streams, lakes and wells also become polluted through the drainage or seepage into them of infected surface washings. The result is that such waters, if used for city water supplies, must be treated so that even occasional pollution, with its dangers of infection, may be guarded against. The water is therefore collected in reservoirs and then is usually filtered, especially if not perfectly clear and clean all the year round. In any case, it must always be disinfected with chlorine, or live pathogenic microorganisms may at some time get into the water pipes and kill not only hundreds but thousands of people. This has happened repeatedly in the past. The small amount of chlorine\* necessary for this purpose is absolutely harmless even though it may at times give a “chemical flavor” to the water.

### FILTER PLANTS

While the construction and operation of apparatus for filtration and disinfection of water are primarily engineering problems, the processes they are

\* Enough must be added to leave a residual of 0.2 to 1.0 mg/L of free Cl.

designed to carry out are based in part on microbiological principles. There are several types of apparatus.

**The Slow Sand Filter.** Large sand and gravel beds, an acre or more in area, are built up over drain pipes, starting with coarse gravel at the bottom and graduating in size to rather fine sand at the top (Fig. 38-1). The water is led onto the sand and allowed to trickle slowly through. The area of the slow sand filter is necessarily large because the water passes slowly through it. As filtration proceeds, day after day, there accumulates, around each grain of sand, and in the interstices, especially in the upper 3 or 4 inches of sand, a slimy, gelatinous film composed of millions of bacteria and protozoa. This slowly closes up the pores in the sand and makes the filter bed more and more effective but also causes the rate of filtration to become slower and slower. At best, slow sand filters yield about 3 million gallons of filtered water per acre per day. The slimy layer is called the "schmutzdecke" (German for "dirt cover"). Through the action of enzymes, biological oxidation and reduction processes, and the ingestion of bacteria by myriads of protozoa inhabiting the slimy film, the bacterial and chemical quality of the water is greatly improved. When the gelatinous film finally becomes too thick, the filter is thrown out of service and the schmutzdecke removed by cleaning machines. A newly cleaned filter is not highly effective until the schmutzdecke begins to form. The effectiveness of the filtration is constantly tested by bacteriologists in the plant who determine the numbers and kinds of bacteria present in the water during different stages of the filtration process, as well as in the finished product. The filters can remove around 99 per cent of the bacteria present in the "raw" water.

**The Rapid Sand Filter.** The rapid sand filter is similar to the slow sand filter in principle, but its area is much less and it does not depend on the growth of a schmutzdecke. It filters water much more rapidly per unit of filter-bed area; about 130 million gallons per acre per day.

In order to obtain rapid filtration of the water it is necessary that most of the foreign material in the water be first removed by some other means. This is usually accomplished by *settling* and *coagulation*. Coagulative substances,

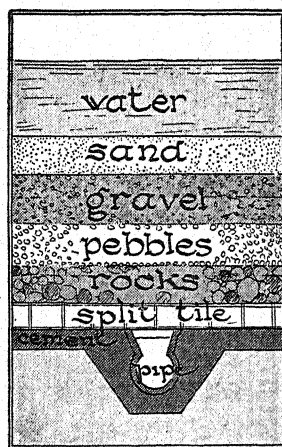


Fig. 38-1. Vertical section of a filter bed. The depth of water varies from 3 to 5 feet above the sand. The principal filtering action is in the upper few inches of sand. The filtered water is drained away in the pipe at the bottom and stored in a cistern. It is chlorinated on the way. (Hunter and Whitman, Problems in General Science, American Book Co.)



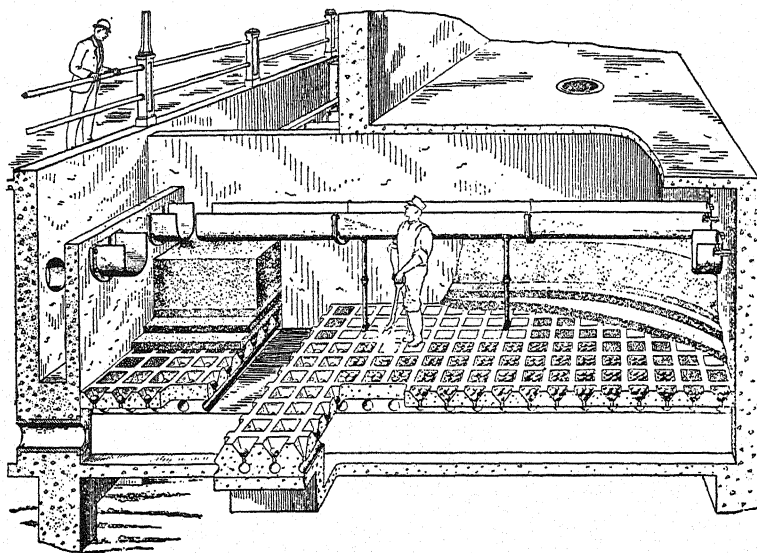


Fig. 38-2. A rapid sand filter, showing, in rear corners, the different layers of sand; under foot, the drains for filtered water and the pipes admitting wash water in a reverse direction; at the digger's shoulders, the troughs leading away the overflow from the washing process. (From Horwood, *The Sanitation of Water Supplies*, courtesy of Charles C Thomas, Springfield, Illinois, publishers.)

sometimes ammonium aluminum sulfate, or a mixture of ferrous sulfate and lime, are added to the water. These form bulky, sticky precipitates which *adsorb* or *occlude* silt, bacteria and coloring matter. The water is held in large settling basins till most of the dirty precipitate has settled out. The sediment is later pumped out from the bottoms of the tanks. The clarified water is run onto the filters. The particles to be removed consist chiefly of the floc remaining after the settling process and, being large, they are readily filtered out by the sand. The bacterial removal is of the order of 99 per cent. The filters remain in service for several hours or days, and then are washed by a flow of water and air forced upward through them. Figure 38-2 shows a rapid sand filter.

In neither process can the filtered water be guaranteed to be free from pathogenic microorganisms. For this reason, in all public water systems in enlightened civilizations, after filtration the water is treated with chlorine and stored in underground cisterns. Highly polluted waters may be made potable, crystal clear and almost sterile by these devices, and it is due largely to these, as well as to improved methods of sewage disposal, that typhoid fever, dysentery and cholera remain at a low level or absent in cities with water filtration plants.

**Combination Process.** In newer processes the extensive basins for coagulation or floc formation and the long time and large tanks required for settling, plus the necessity of loading filters with the remains of unsettled floc, and elaborate washing apparatus, are to a great extent overcome. *Tank machines* (often called "package plants") are arranged in which large, heavy particles of floc are built up by chemical action between the coagulating chemical and

the raw water in a *mixing and reaction zone*. Paddles provide *vigorous circulation* of the coagulating chemicals and the raw water in a "slurry pool." The heavy floc particles actively adsorb color, microorganisms, and finely divided suspended matter. Being large and heavy they rapidly settle out as sediment in the lower parts of the slurry pool. Excess sediment is removed in a "concentrator" or drain. The supernatant water is quite clear and is led off to be finally filtered, chlorinated and stored in a covered reservoir. There are various engineering devices to accomplish these purposes.

### SEWAGE PURIFICATION

The "purification" of sewage in cesspools and municipal sewage-disposal plants is almost wholly dependent on processes in which microorganisms play the chief parts. The processes are, fundamentally, the same as those occurring in natural decay and putrefaction, but are controlled and exploited to the best advantage by the sanitary engineer. Many of the species of water, soil and intestinal bacteria already mentioned are involved in both processes. *Aerobic* action and *oxidative* processes are promoted especially in the fluid parts of sewage, while anaerobic processes occur in digestion of sludges, except activated sludge, which is aerated.

Most cities have separate sewerage systems to drain off storm waters. Industrial wastes (from dairies, tanneries, paper mills, etc.) are usually disposed of in plants specially designed for the particular material involved. Such wastes often require rather complex preliminary chemical treatment, but biological oxidative action is usually the final "purifying" agent. Such materials are often led into broad, shallow, open, artificial "oxidation ponds" or lagoons, there to undergo (partly) aerobic, oxidative processes. As an interesting sidelight on conservation of food and utilization of solar energy (a pet subject for engineering research), experimental attempts are being made to cultivate algae (*Chlorella*, *Scenedesmus*) in such ponds, fully exposed to sunlight, on a large and intensive scale. Protein, a very expensive stock food, may thus be produced cheaply from sewage and sunlight, resulting eventually in spare ribs and omelets on your table.

If "raw" domestic or "sanitary" sewage is discharged directly into natural bodies of water, the available oxygen in the water is soon used by the microscopic organisms living in the sewage, and foul-smelling, anaerobic processes develop and create a nuisance. Further, aquatic life is impossible, and considerable economic loss results. Still further, the water is ruined for drinking, swimming or esthetic purposes.

**Composition of Sewage.** The sewage of a city is about 99 per cent water, and is handled in pumps and tanks much as is drinking water.

Fresh city sewage contains dilute excrement, along with other city wastes: comminuted garbage, laundry water, and the like. Consequently it contains the flora and fauna of the intestinal tract, as well as many soil and water species, and much organic matter. The pH usually ranges around the neutral point; temperature varies seasonally (5–25° C). Of the solid material (after removal of silt, gravel, bottles, etc.) nearly half is cellulose.

**Microorganisms in Sewage.** These include many *aerobes*, strict anaerobes and facultative anaerobes, mostly saprophytic heterotrophs. Common types of bacteria from soil and intestine are: Enterobacteriaceae, enterococci,

*Clostridium*, *Bacteroides*, *Cytophaga*, *Micrococcus*, *Pseudomonadaceae*, spirochetes, *Lactobacteriaceae*, *Achromobacteriaceae*, sulfur bacteria, iron bacteria, yeasts, molds and actinomycetes.

*Sphaerotilus*, *Crenothrix*, *Beggiatoa*, and probably filamentous *Rhodobacteriineae*, (if sunlight is present) characteristically form slimy growths on the sides and bottoms of sewage-containing ditches, pipes, tanks, etc. They are often called "sewage fungi." Certain real fungi (Phycomycetes), *Saprolegnia* and *Leptomit*us, are often found among them. All of the organisms aid in decomposition of organic matter in the sewage.

**Changes in Sewage.** Not all of these organisms continue to thrive. The pH changes toward acidity due to fermentation, and acid-sensitive species disappear. Unless air is vigorously bubbled through the sewage (a common procedure today) the oxygen dissolved in the water is soon used up and the O-R potential becomes strongly negative. The *biological oxygen demand* (commonly called BOD) increases. The strict aerobes cease functioning. Only facultative and strict anaerobes can thrive. Being unable to use oxygen to cause complete oxidations of organic matters they cause partial decomposition of organic compounds, with formation of odoriferous volatile compounds: various sulfur amines, butyric acid, hydrogen sulfide and the like. A great nuisance is created. The sewage is said to be in a "septic" condition.

Biologically, a kaleidoscopically changing picture is presented; one physiological type succeeds another in rapid succession as conditions in the sewage develop, to which each successive form is peculiarly adapted and to which the preceding form was not. There is also a complex interplay and competition between various bacteria, and between them and protozoa and other small animal forms which thrive mightily in sewage.

**Biological Actions.** As we have seen, many of the common saprophytes of soil, water and the intestinal tract possess marked powers of hydrolysis. Proteolytic forms bring about digestions of proteins; lipolytic forms hydrolyze fats and related compounds; nearly all forms can hydrolyze one or more carbohydrates. The woody vegetable materials in sewage are decomposed by numerous species capable of hydrolyzing cellulose. A piece of linen or fabric or a thick sheet of cellulose filter paper will be digested and disappear completely in active sewage in 5 to 7 days; faster in warm weather or heated sewage. Still other forms hydrolyze all manner of organic matter: phenol rubber, paraffin, and so on. Some of these organisms have been mentioned. Others, especially certain gram-negative, anaerobic, cellulose-digesters of sludge, are still imperfectly known.

**SYNTROPISM.** The products of hydrolysis by one organism\* are excellent pabulum for still another species, and so on. This is a form of *syntropism*. The original molecules of wood, fat, meat, etc., are finally changed, usually through the combined actions of several species, into soluble, relatively simple substances.

✓ **AEROBIC PROCESSES.** Under aerobic conditions all are eventually changed into sulfates, phosphates, ammonium salts, nitrates, CO<sub>2</sub>, H<sub>2</sub>O ("mineral-

\* Say, for example, starch is hydrolyzed to erythrodextrin, or cellulose to cellobiose, or fat to glycerol and fatty acid, or flesh protein to albumoses.

ized"). Many of these compounds are rebuilt eventually into crop plants; these into animals and these in turn, dear reader, into you and me!

**ANAEROBIC PROCESSES.** If the digestive process is anaerobic (as in tank-digested sludge) reduced compounds are formed, some of which were listed earlier:  $H_2S$ ,  $N$ ,  $NH_3$ ,  $CH_4$ , etc. Others are products of partial decomposition. These, when transferred to soil or bodies of water, are utilized as sources of energy by the saprophytic and autotrophic microorganisms capable of oxidizing them (*Thiobacillus*, *Nitrobacteriaceae*, *Methanomonas*, *Rhodobacteriineae* and the like). In the oxidized form (sulfates, nitrates, carbonates, etc.) they are available to higher plants as noted above, and so the cycle runs.

**Importance of Oxygen.** It is evident that, throughout the whole process, oxygen content of the water is the principal controlling factor. If thorough oxidation could be maintained throughout, the offensive, anaerobic, septic condition would not occur and complete, oxidative decomposition would proceed much more rapidly, with little or no nuisance. Such is the object of modern sewage disposal plants.

#### SEWAGE DISPOSAL PLANTS

Sewage disposal plants (Fig. 38-3) are operated to accomplish several ends as follows:

1. *Screening*; to remove bulky foreign matter such as grit, bottles, paper, wooden boxes and other extraneous refuse as well as allow grit and gravel to settle out.
2. *Separation* by gravity of all organic matter in the sewage which will settle to the bottom of tanks (settleable solids) or float on the surface (scum).
3. *Aeration* and biological treatment of the supernatant fluid and usually final chlorination of the supernatant fluid.
4. *Digestion, and drying* of the sludge or sediment.
5. *Collection of gas* from sludge digestion.
6. *Disposal of the sludge.*

Numerous methods have been devised for accomplishing these various ends. The first is largely a matter of mechanics and does not concern us. The second is of interest since it is here that microbial action first comes into play.

Various kinds of *preliminary settling* or sedimentation tanks are employed, all designed to allow the solid matter to settle out as much as possible, and in which the sewage is held for some time (usually from 2 to 10 hours). It is a modification of the natural process of sedimentation that goes on constantly in rivers, lakes and the ocean. Flotation may also occur (fat, wood, etc.) and surface-skimming is often an important part of preliminary settling. From 40 to 60 per cent of the solid matter of sewage settles out of suspension as sludge in 3 hours in these tanks. Sedimentation is sometimes hastened by the addition of flocculating agents similar to those used in water purification.

The sludge, of which about 95 per cent or more is water, is drawn off from the depths of the tanks through pipes and is transferred to sludge-digestion tanks where slow, mostly anaerobic, decomposition continues. Here the sludge may be held for weeks or months. It is finally transformed to a dark, viscous fluid in which little or no further biological action can occur. It is removed from the tank and disposed of on sludge-drying beds or it may be

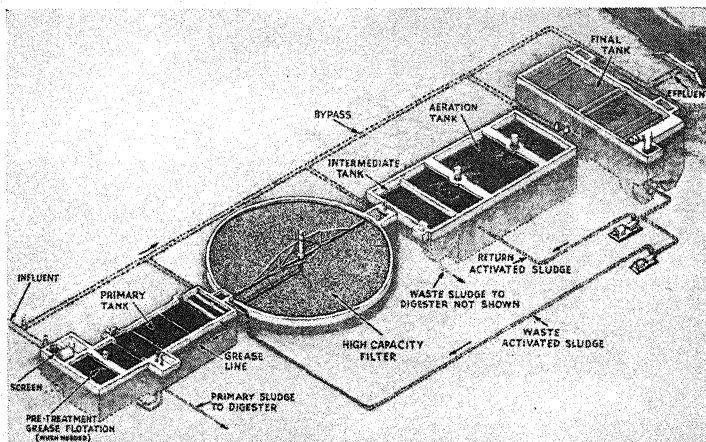


Fig. 38-3. A modern municipal process of sewage purification combines the best features of activated (aerated) sludge, settling tanks and sludge digestion. It gives maximum biologic decomposition of the organic matter in the sewage in minimum space and time. The large round structure is a gravel bed filter (see Fig. 38-5) over which the fluid is sprayed by the rotating arms. This permits greater aeration and speeds up the microbial action on the sewage. (Courtesy of The American Well Works, Aurora, Ill.)

The sewage enters at the influent (left), is screened and then passes through preliminary settling tanks where solids settle to the bottom as sludge and grease floats on the surface. The sludge is removed for anaerobic digestion. The partly clarified sewage is then passed over the aerated, high-capacity filter. Passing then through an intermediate tank any remaining solids settle out and are taken off for anaerobic digestion. The supernatant fluid then is thoroughly aerated with activated sludge and flows into the final tank where the activated sludge particles settle out and are returned to the aeration tank. The fluid is then chlorinated and passed out through the effluent into a river or other adequate body of water. When too much activated sludge accumulates part is put into the regular sewage for routine disposal (waste activated sludge). Pipelines are available for bypass of any part of the system or, indeed, the entire disposal plant in case of breakdown or emergency.

de-watered in large heated drums, and pulverized, incinerated or sold as fertilizer. "Milorganite" is a good example.

**"Two-Story" Tanks.** Some tanks are made in two compartments, an upper and a lower. The upper portion is like a long, double, V-shaped trough and serves to introduce fresh sewage (Fig. 38-4). The solid matter settles out through slots at the bottom of the V's into a deep sludge-slump as the fluid flows along the trough. The solid matter, after settling to the lower compartment, is held and stored, digestion and decomposition going on there. The sludge may be removed to other digestion tanks. Gases from the sludge compartment are collected and led to the surface by a funnel-shaped structure inverted over the sump. This gas contains about 75 per cent methane produced by reduction of carbon by anaerobic bacteria. It is collected in gas tanks and used as fuel. It often serves to furnish steam and electric power for most of the light, heat and power requirements of the disposal plant. This energy was once the energy of life! The digested sludge, largely mineral residue, is pumped out from the bottom at intervals. The best known tank of this type is the *Imhoff tank*.

**Aeration and Disposal of Fluid.** The fluid part of the sewage, after passage through the sedimentation tanks or above the sludge compartment of the

Imhoff tank, still contains much putrescible organic matter. It is often passed through a *secondary clarifier* or settling tank. The clarified fluid may then be disposed of, where a porous and dry soil is available, by surface ditches or by a subsurface irrigation system of tile pipes, furnishing excellent fertilizer for farm crops raised on the land. There is probably little danger of infection as typhoid, dysentery, and cholera organisms are largely killed, either by the antagonistic action of saprophytic bacteria in the settling tanks or soil, or are filtered out by the soil and die. However, tubercle bacilli, *Salmonella* and polio virus have been isolated from Imhoff-tank effluent, as well as from secondary effluents, so that promiscuous use of undisinfected sewage effluents is not entirely safe.

**AERATING FILTERS.** The fluid from any form of settling tank may also be allowed to drain through artificial beds of sand, or broken stone, the filtrate being collected by under-drains much as in slow sand filtration of water. This process of purification is similar to those used in filtering drinking water, the sand, grains or pieces of stone becoming coated with a living film of aerobic, strongly-oxidative microorganisms much like *schmutzdecke*. These feed upon, and oxidize, the organic matter of the sewage; the result being a much less offensive liquid. Since the fluid is initially very poor in oxygen, due to the BOD of the organisms already in it, it must be well *aerated* to enhance aerobic oxidation of organic matter and prevent foul-smelling anaerobic action. For this reason such filters require draining and aeration daily to allow oxidation processes to occur. They are, therefore, *intermittent* in operation.

**TRICKLING FILTERS.** A more effective type of aerating device, operating on similar principles, is the *trickling filter* in which the fluid is intermittently sprayed over the stones by stationary nozzles or rotating spargers instead of

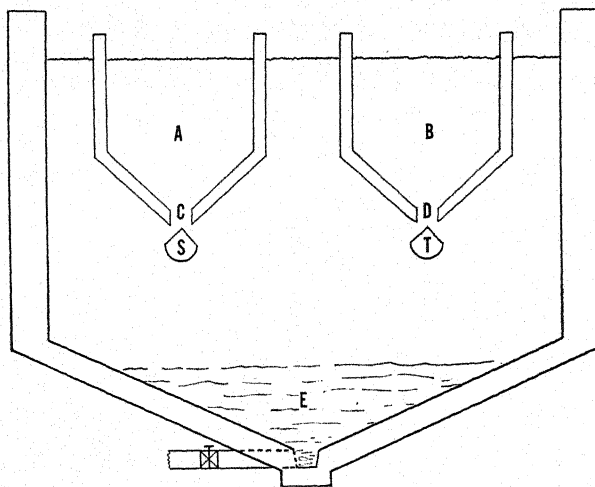


Fig. 38-4. Structure and operation of an Imhoff-type sewage-clarification tank. The tank may be 10 by 20 feet in dimensions and perhaps 10 feet deep. The diagram shows a cross section. Sewage flows slowly (vertically to the page in this diagram) through the troughs A and B. Solids settle through slots C and D to the sludge sump at E. Sludge is drawn off periodically through the drain pipe to a sludge-digestion tank. Gas bubbles rise around the baffles at S and T. Scum is removed at the surface as it forms.

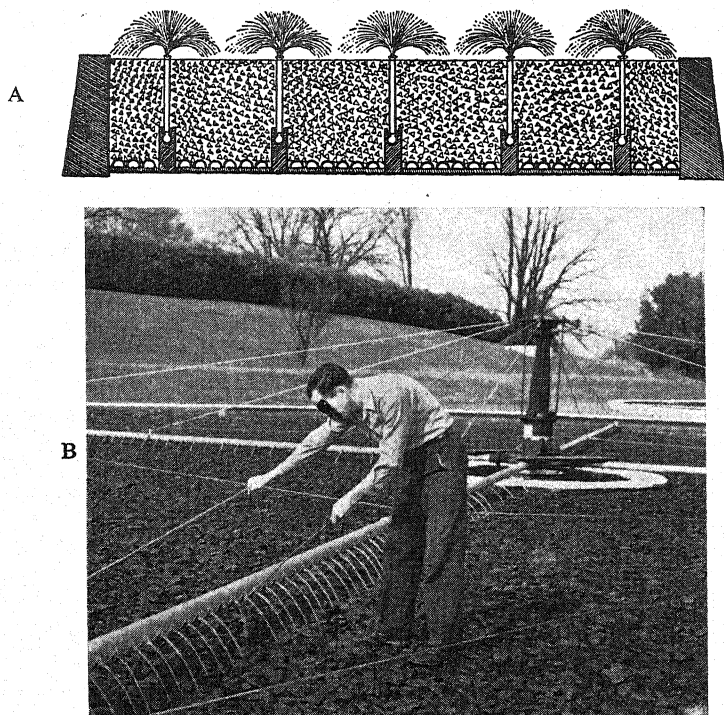


Fig. 38-5. Types of trickling filter. At *A* is seen a concrete tank filled with coke or broken stone over the aerated surfaces of which the sewage is sprayed through nozzles. In an older manner of use the tank is alternately filled with, and emptied of, sewage; a process which intermittently aerates the actively oxidizing film of growth on the surfaces of the coke or stones. At *B* is seen a more modern use of the same principle. The rotating sparger applies the fluid intermittently. In this picture the sparger has been stopped to clean a plugged nozzle. Note the dark-colored growth on the surfaces of the stones. (*A*, courtesy of Dr. Gordon M. Fair in Rosenau, Preventive Medicine and Hygiene, D. Appleton-Century Co.; *B*, courtesy of Communicable Disease Center, U. S. Public Health Service, Atlanta, Ga.)

alternately flooding and draining them (Fig. 38-5). The fluid adsorbs oxygen as it is sprayed through the air, and the interstices between the stones are filled with air. Oxidation is rapid and complete.

**Activated Sludge.** If air is bubbled actively through sewage in a tank, aerobic conditions are maintained throughout the liquid. Particles of suspended matter flocculate, after a time, into small masses swarming with aerobic microscopic life and capable of oxidizing organic matter readily. These masses are called "activated sludge." As the aeration process continues, the volume of the floc, or *activated sludge*, increases as more and more sewage is passed through the tank.

**ACTIVATED SLUDGE ORGANISMS.** The particles of floc in activated sludge consist of mixed species of bacteria which embed themselves in a mass of polysaccharide gum called "zooglea" (more properly, phytoglea). One of the principal zooglea-forming species is a *Pseudomonas*-like species called



*Zoogloea ramigera*. Numerous other familiar microorganisms can also form (or help to form) zoogloal masses under the conditions of activated sludge: *Escherichia*, various *Pseudomonas*, *Alcaligenes*, *Bacillus*, etc. Floc is formed by the adherence together, in clumps, of several bacterial cells at their capsular surfaces.

The sticky zoogloal material gathers up, by adhesion and by adsorption, much of the colloidal material, bacteria, color and odors of the sewage fluid.

After a good, active floc of activated (aerated) sludge is formed in a tank, more sewage can be passed through the tank and rapid clarification, organic decomposition, oxidation, and decrease in bacterial content is obtained, provided aeration continues and enough activated sludge is intimately mixed with the traveling sewage. The basic principle of the process is the same as that of aeration and filtration, the sand grains or stone of sewage filters being replaced by the living particles and air in the activated sludge.

The fluid part of the mixture is afterward passed into a final settling tank or clarifier, or trickling filter, part of the active, solid sludge being retained in, or returned to, the aeration tank as "seed."

**Rapid Methods of Sewage Purification.** It is clear that *rapid* and *complete* oxidation and decomposition of the organic matters in sewage depend primarily on vigorous growth of aerobic microorganisms and this, in turn, on the presence of ample food and oxygen. Machines have been devised in which air may be mixed with the sewage in the sewers before it even reaches the disposal plant. This provides a well-oxidized sewage to begin with. In other systems (after screening and removal of grit), raw sewage is immediately mixed with activated sludge and *vigorously* and *continuously aerated* by paddles in the depths of a specially designed tank. The machines are built and operated on the same principle as the "package" water filtration plants described in a foregoing section.

#### PREVENTION OF WATER-BORNE DISEASE IN ABSENCE OF FILTRATION

Disease transmission by water in the absence of elaborate filtration and chlorination systems, as on camping trips, is easily prevented. All of the common intestinal pathogens are readily killed by boiling for five minutes as well as by contact for at least 2 hours with chlorine, iodine, or other suitable disinfectants.

Ice made from polluted water is nearly as dangerous as the water itself.

Water for camp use, provided it is clear and clean, may be treated with chlorine by using one of the methods of field chlorination involving tablets of  $\text{CaOCl}_2$  available on the market. A teaspoonful of fresh chloride of lime in 25 to 50 gallons of water will also prove an adequate safeguard unless the water is very heavily polluted or dirty. At least an hour must be allowed for the chlorine to act. Ordinary laundry bleaches ( $\text{Na}_2\text{OCl}$ ) will also serve. Directions are found on the labels of the bottles. If you are contemplating a camping trip, or travel in countries where water and sanitation are poor, write for Public Health Service Publication No. 387, "Drinking Water Disinfection," 1954, Superintendent of Documents, Government Printing Office, Washington 25, D.C. (5 cents).



## REFERENCES

- Babbitt, H. E.: Sewerage and Sewage Treatment. 7th ed. John Wiley and Sons, Inc., New York, 1953.
- Drinking Water Disinfection. Pub. Health Serv. Publication No. 74, 1954. Sup't Doc., Washington 25, D. C.
- Editorial: Infectious agents in sewage. J.A.M.A., 1956, 160:877.
- Ehlers, V. M., and Steel, E. W.: Municipal and Rural Sanitation. 4th ed. The McGraw-Hill Book Co., New York, 1950.
- Fair, G. M., Geyer, J. C., and Morris, J. C.: Water Supply and Waste Water Disposal. John Wiley and Sons, Inc., New York, 1954.
- Gainey, P. L., and Lord, T. H.: Microbiology of Water and Sewage. Prentice-Hall, Inc., New York, 1952.
- Gottas, H. B.: Composting, sanitary disposal and reclamation of organic wastes. World Health Org. Monogr. Ser. No. 31. Columbia University Press, New York, 1956.
- Hardenbergh, W. A.: Sewerage and Sewage Treatment. 3rd ed. International Textbook Co., Scranton, Pa., 1950.
- Heukelekian, H.: Microbiology of water and sewage. Ann. Rev. Microbiol., 1953, 7:461.
- Heukelekian, H.: Stabilization of polluted waters. Water and Sewage Works J., 1955, 102:82.
- Individual Water Supply Systems, 1950. Public Health Serv., Publication No. 24. Sup't Doc. Washington 25, D. C.
- Maki, L. R.: Experiments on the microbiology of cellulose decomposition in a municipal sewage plant. A. V. Leeuwenhoek J. Micr. and Serol., 1954, 20:13.
- McKinney, R. E., and Weichlein, R. G.: Isolation of floc-producing bacteria from activated sludge. Appl. Micr., 1953, 1:259.
- Odum, E. P.: Fundamentals of Ecology. W. B. Saunders Co., Philadelphia, 1953.
- Renn, C. E.: Algae research on oxidation ponds. Am. J. Pub. Health, 1954, 44:631.
- Van Kleeck, L.: Fertilizer value in waste disposal methods. Am. J. Pub. Health, 1954, 44:349.
- Wang, W. L. L., Dunlop, S. G., and De Boer, G.: The survival of *Shigella* in sewage. Appl. Micr., 1956, 4:34.
- Warrick, L. F., Chairman, Engineering Section. Package Sewage Treatment Plants, and Special Disposal Problems. Am. J. Pub. Health Year Book, Pt. 2, 1952.
- Weibel, S. R., Bendixen, T. W., and Coulter, J. B.: Studies on household sewage disposal systems. I, II, III. Pub. Health Service Publ. No. 397. Gov't. Printing Off., Washington 25, D. C., 1955.

## Some Pathogenic Gram-Negative Rods: Enterobacteriaceae; Parvobacteriaceae

### THE PATHOGENIC ENTEROBACTERIACEAE

SOME OF the lesser, occasionally pathogenic Enterobacteriaceae were mentioned in the previous chapter: some varieties of *Escherichia coli*; some groups of *Paracolobactrum*; and occasionally *Proteus*. The most important pathogens of the family are comprised in the genera *Salmonella*\* and *Shigella*.\*

The *Salmonella* and *Shigella* are typical Enterobacteriaceae in all morphological and physiological respects, differing from those discussed in the last chapter mainly in: (a) being more pathogenic; (b) failing to ferment lactose or sucrose (there are one or two important exceptions). *Salmonella* are motile; *Shigella* are non-motile. The genera most likely to be confused with *Salmonella* because of slight, slow or absent fermentation of lactose are *Proteus* and *Paracolobactrum*. These genera are readily differentiated by the physiological characters shown in Table 24.† All of the Enterobacteriaceae are closely allied, as shown by the fact that antigenic relationships are commonly found between species in all genera in the family. Further, some "species" have identical biochemical properties. Consequently, in identifying species, it is frequently necessary to go through a complex serological process called *antigenic analysis*, using adsorbed sera (Chapter 21), before the true status of an organism is known; even then the position of many is left in doubt.

### GENUS SALMONELLA

The salmonellas cause infections (commonly called salmonellosis) in man and many species of domestic and wild animals. The resulting diseases are characteristically gastrointestinal, but may be completely generalized in the body, and may range in severity from almost imperceptible intestinal dis-

\* The apparently meaningless generic names *Salmonella* and *Shigella* are derived from names of famous bacteriologists. Salmon was an American scientist, noted for his work on hog cholera (1885). Shiga was a Japanese bacteriologist who first determined the cause of highly fatal epidemics of dysentery in Japan in 1896.

† It must be understood that, in describing any group of bacteria, allowance must be made for variation and mutation. Forms of any species not infrequently occur which are aberrant with respect to any physiological characteristic.

Table 24. Differential Characters\* of Important Non-lactose-fermenting (or slow-lactose-fermenting) Enterobacteriaceae.

ORGANISMS	INDOLE PRODUC- TION	FERMENTATIONS OF						HYDROLYSIS OF		MOTIL- ITY	UTIL- IZE CIT- RATE**	GROWTH IN KCN BROTH	H <sub>2</sub> S IN TSI AGAR
		LAC- TOSE	GLU- COSE (with gas†)	SUC- ROSE	ADON- ITOL	DULCI- TOL	MANN- ITOL	UREA	GELA- TIN				
<i>Salmonella</i>	-	-	++	-	-	+	+	-	-	+	§	-	+
<i>Shigella</i>	+ or -	-†	-	-†	-	+ or -	+ or -	-	-	-	-	-	-
<i>Paracolobactrum</i> — Arizona	-	+ or -	+	-	-	-	+	-	+	+	+	-	+
<i>Paracolobactrum</i> — Providence	+	-	+	-†	+	-	-	-	-	+	+	+	-
<i>Paracolobactrum</i> — Bethesda, Ballerup ( <i>Esch. freundi</i> )	-	-†	+	+ or -	+ or -	+ or -	+	+ or -	-	+ or -	+	+	+
<i>Proteus</i>	+ or -	-	+ or -	+ or -	+ or -	-	+ or -	+	+ or -	+	+ or -	+	+ or -

\* Reactions of special differential value are in **bold face**.

† Some species slowly positive.

‡ Gas in glucose media implies gas in other fermentative tests.

\*\* As sole source of carbon in otherwise inorganic medium.

|| *S. typhi* produces acid only; no gas.§ Usually; *S. typhi* is negative.

comfort to fatal disease. The habitat of the organisms is mainly the intestinal tract and tissues of infected animals, but the organisms can grow in feces-polluted foods and may survive in polluted or infected foods, waters and on fomites for periods of from a few hours to days.

Of the salmonellas, *S. typhi* (the cause of typhoid fever), and the so-called "food-poisoning" or paratyphoid group: *S. paratyphi A*; *S. paratyphi B*; *S. paratyphi C*; *S. typhi murium*; *S. enteritidis*; and *S. cholerae-suis* are among the most important. There are over 300 named types (serotypes), including the above.

**Isolation of Salmonella** (also *Shigella* and *Paracolobactrum*) from feces, foods, tissues, etc., is greatly facilitated by selective cultivation. Media used for this purpose contain: (a) a nutrient agar or broth base; (b) agents to inhibit unwanted microorganisms; (c) lactose and some acid-indicator dye (like litmus or phenol red) to reveal and differentiate the lactose-fermenting, acid-producing, non-pathogenic organisms (colonies are colored by acid indicator). The non-lactose-fermenting *Salmonella*, *Shigella* or *Paracolobactrum* colonies are colorless. Numerous selective inhibitory agents are used: sodium desoxycholate; fuchsin-sulfite mixtures combined with eosin and methylene blue; sodium selenite; sodium tetrathionate; brilliant green, etc. There are scores of such media. All are based on the same principles. The steps are outlined in Chart 2.

**Chart 2. Outline of Procedure for Preliminary Isolation and Identification of Pathogenic Enterobacteriaceae.**

- I. Sample is inoculated onto plates of selective agar media:
  - (a) Eosin-methylene-blue agar
  - (b) Desoxycholate agar
  - (c) Bismuth sulfite agar (especially good for *S. typhi*)
- II. Sample is inoculated into tube of broth containing selective agents:
  - (a) sodium selenite
  - (b) sodium tetrathionate

II'. After incubation of selective broths about 18 hours, plate out as in step I, above.
- III. After overnight incubation of all plates inoculated as indicated above, pick suspected colonies to:
  - (a) TSI agar slants (detect gas, acid and  $H_2S$ )\*
  - (b) Urea medium to detect urease formation.
- IV. If, after 24 hours of incubation, TSI agar shows reactions of *Shigella* or *Salmonella* (Fig. 39-1), try agglutination with polyvalent *Salmonella* or *Shigella* serum.
- V. Carry out additional tests for motility and various enzymic activities (Tables 23, 24, 25 and 26).

**Antigenic Analysis.** With a collection of adsorbed, monovalent agglutinating sera (Chapter 21), each representing a single, different, antigenic component of the various species of *Salmonella*, one may test any given organism for the presence of different antigenic components and assign to it a "formula" expressing the antigenic complex of which it is composed. This sort of antigenic analysis has been developed in the genus *Salmonella* to

\* TSI medium contains lactose, glucose and sucrose, with an acid indicator, and an iron salt ( $FeCl_3$ ) to turn black ( $Fe_2S_3$ ) if  $H_2S$  is formed. The medium is used in the form of slants in culture tubes and gives characteristic reactions depending on the species inoculated into it (Fig. 39-1).

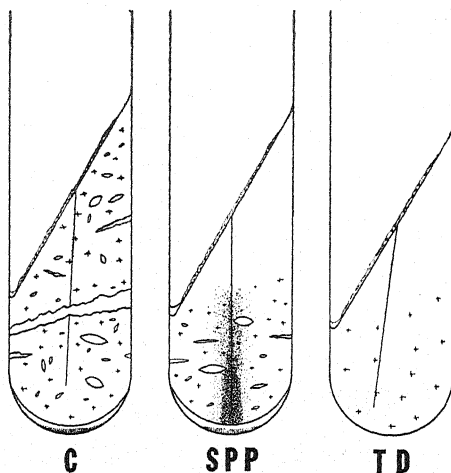


Fig. 39-1. Cultures of Enterobacteriaceae in an agar medium designed to differentiate major groups. The medium is triple-sugar-iron (T-S-I) agar. It contains lactose, glucose and sucrose with acid-alkali indicator.  $\text{FeCl}_3$  is added to detect the formation of  $\text{H}_2\text{S}$ . The slants are inoculated on the surface and by a stab of the needle into the depths of the butt of the agar. *C* shows the reaction of coliform organisms: gas bubbles and acid (crosses) throughout the agar. Note that the volume of gas formed has rent the agar slant at several places and pushed the butt of the agar slant away from the bottom of the tube, where a few drops of bacterial suspension have collected. *SPP* shows the reaction typical of *Salmonella* organisms and also of some strains of *Proteus* and *Paracolobactrum*: acid and gas, with formation of  $\text{H}_2\text{S}$  (shading) in the butt; alkaline slant (upper portion). *TD* shows the reaction of *Salmonella typhi* and of the genus *Shigella* (typhoid-dysentery): acid butt, alkaline slant; no gas, no  $\text{H}_2\text{S}$ . Further differentiations are made on the basis of reactions shown in Tables 24, 25 and 26 and serological tests.

a high degree and we may now state the antigenic formula for each of some 300 or more "species" or serotypes.

**KAUFFMANN-WHITE SCHEMA.** By means of such antigenic analyses the salmonellas have been arranged in a series often referred to as the "Kauffmann-White schema." In this schema the O (somatic) antigens are given Arabic numbers. The flagellar (H) antigens have 2 series of numbers, depending on *phase variations*: \* small Roman letters if in phase I; Arabic numbers if in phase II. The antigenic structure of any given species may therefore be expressed in terms of these numbers and letters. For example, *S. typhi murium* has the antigenic formula, 1, 4, 5, 12: i; 1, 2 (see Table 27). Species having one or more *somatic* antigens in common are placed in convenient groups: A, B, C, etc.

Similar antigenic schemes are found in other groups of bacteria: *Clostridium*, *Shigella*, *Klebsiella*, *Corynebacterium*, *Escherichia*, etc.

\* In many cultures of *Salmonella* there appear two types of cells. Some of the cells are in what is called the "specific phase" (phase I). In this phase they agglutinate best with H serum specific for *only* that species. Other cells in such *diphasic* cultures are in the "group phase" (phase II). In this phase they agglutinate to a considerable extent in H sera for other, closely related, species. Sometimes the culture may be almost entirely in phase I; at other times predominantly in phase II. Some cultures do not alternate phases; i.e., they are *monophasic*.

Table 25. Differential Properties of Some *Salmonellas*.\*

SPECIES	XYLOSE	TREHALOSE	ARABINOSE	H <sub>2</sub> S	CITRATE ASAR	GAS IN DEXTROSE
<i>S. typhi</i>	V	+	—	+	—	—
<i>S. paratyphi A</i>	—	⊕	⊕	—	—	+
<i>S. paratyphi B</i>	⊕	⊕	⊕	+	+	+
<i>S. paratyphi C</i>	⊕	⊕	⊕	+	+	+
<i>S. enteritidis</i>	⊕	⊕	⊕	+	+	+
<i>S. typhi murium</i>	⊕	⊕	⊕	+	+	+
<i>S. cholerae-suis</i>	⊕	—	—	—	+	+

\* ⊕ = acid and gas; + = positive test or gas only; V = variable

Table 26. Some Cultural Reactions of the *Shigellas*.\*

SEROLOGICAL GROUP	PRINCIPAL SPECIES	MANNITOL	LACTOSE	SUCROSE	INDOLE PRODUCTION
A	<i>Shigella dysenteriae</i>	—	—	—	—
	<i>Shigella ambigua</i>	—	—	—	+
B	<i>Shigella flexneri</i>	+	—	—	±
C	<i>Shigella boydii</i>	+	—	—	±
D	<i>Shigella sonnei</i>	+	Slowly +	+	—

\* There are exceptions to most of the reactions, in aberrant strains.

Table 27. Antigenic Formulae of Some *Salmonella* Species.

GROUP	SPECIES	O ANTIGENS	H ANTIGENS	
			Phase I	Phase II
A	<i>S. paratyphi A</i>	1, 2, 12	a	...
B	<i>S. paratyphi B</i>	1, 4, 5, 12	b	1, 2
	<i>S. typhi murium</i>		i	1, 2
C	<i>S. paratyphi C</i>	6, 7, Vi*	c	1, 5
	<i>S. cholerae-suis</i>	6, 7	c	1, 5
D	<i>S. enteritidis</i>	1, 9, 12	g, m	...
	<i>S. typhi</i>	9, 12, Vi	d	...
E	<i>S. senftenberg</i>	1, 3, 19	g, s, t	...

\* Vi antigen was formerly thought to confer virulence on *S. typhi* (acting somewhat like a capsule). It is now known to occur regularly in several other virulent Enterobacteriaceae. It also occurs in some nonvirulent forms (so confusing!). Vi stands for virulence.

**Names of *Salmonella*.** Many antigenic types of *Salmonella* closely resembling the major species named above, differing perhaps in only one antigenic component, or one biochemical character, have been given species names. Some of these names are derived from the place where the organism was found, as, for example, "senftenberg," "newport," "kentucky" and "mississippi." There is a tendency now to omit such names and use simply antigenic formulas of the various serotypes.

**Variation of Species.** A confusing feature of this system is the fact that, by various procedures, including transduction, one type may readily be changed into another! Undoubtedly such changes also occur in Nature. New types are constantly being found.

**Salmonellosis.** *Salmonella*, like all of the Enterobacteriaceae, are transmitted by feces or urine, or both, of patients or carriers. The most common vector for any or all of them is soiled hands. Another is food which has become infected and allowed to stand in a warm place, after little or no cooking, so that the organisms can grow. These organisms grow well at warm room temperatures (65° to 100° F). Infection of the food is sometimes by unwashed hands of a very mild case, or human carrier.

*Infection of food* may be by introduction of excrement of dogs, mice or rats which harbor, particularly, *S. typhi murium* as well as other salmonellas. So-called "meat-poisoning" often results from eating or handling raw or improperly cooked flesh of cattle, swine, poultry, fish, or other animals suffering from infection with these organisms, especially *S. cholerae-suis* and *S. enteritidis*. These species are among the most commonly-occurring in this country. Animals of many sorts harbor a variety of *Salmonella* species. Even hens' eggs are often infected, before being laid, by maturation in an infected hen. The eating of raw eggs or egg products is therefore not wise, and has frequently resulted in large outbreaks due to mayonnaise in sandwiches, etc.

Obviously, avoidance of these diseases means cleanliness in the kitchen; sanitary habits on the part of food handlers; care to see that food is properly cooked to kill all organisms, even those in the center of large masses; proper refrigeration of stored food; and avoidance of uncooked foods at club suppers or on picnics, often prepared during the morning or previous evening and then unwittingly *incubated in the kitchen* or in transit. *Salmonella* food infections are very common. Characteristically the onset is at least 12 (usually 18 to 24) hours after eating the infected food. The bacteria multiply during this "incubation period."

**TYPHOID VACCINATION.** Probably many of my readers will have received typhoid "shots" at some time before studying microbiology, possibly on entering the armed services. These injections are a good example of a method of active artificial immunization. The material usually injected is saline solution containing about one billion *Salmonella typhi* per ml killed by heating at about 65° C for 30 minutes, or with formaldehyde. Often included, also, are killed *S. paratyphi A* and *S. paratyphi B*.

For initial immunization, 3 doses (0.5 ml, and 1.0 ml) at weekly intervals are required. Revaccination with small doses (0.1 ml intracutaneously or 0.5 ml subcutaneously) annually is recommended in order to maintain immunity at an effective level. This is a good example of the use of the secondary antigenic stimulus or "booster dose" (see Chapter 21).

## GENUS SHIGELLA AND BACILLARY DYSENTERY

The organism discovered by Shiga in 1896 during a frightful epidemic of dysentery in Japan, with over 22,000 fatalities, is now called *Shigella dysenteriae*. It is type species of the genus. The principal distinguishing characteristics of the genus are shown in Table 24. After Shiga's discovery many other kinds ("species") of dysentery bacilli were discovered by Flexner, Boyd, Sonne and others. The classification and differentiation of these species present problems analogous to those related to classification of the salmonellas (Tables 26 and 27).

**Bacillary Dysentery.** The shigellas cause intestinal disturbances ranging from very mild diarrhea to severe and sometimes fatal dysentery with intense inflammation and ulceration of the large bowel, often with scar formation and stricture of the bowel after recovery. Unlike *Salmonella typhi*, which always causes bacteriemia, *Shigella* do not commonly invade the blood stream. In some epidemics of bacillary dysentery, especially those due to *S. dysenteriae*, the fatality rate is high.

The transmission and prevention of bacillary dysentery are similar to those aspects of salmonellosis, except that animals do not commonly transmit dysentery.

**DYSENTERY VACCINATION.** Vaccination against bacillary dysentery appears to be much less satisfactory than typhoid vaccination.

## PATHOGENIC ESCHERICHIA

If given a large enough dosage and sufficient opportunity, such as a very dirty wound or an old, slowly healing ulcer, *Escherichia coli* may at times cause disease, especially if the patient's general health and non-specific resistance are low. *Escherichia coli* may cause more serious trouble by invading the bladder and pelvis of the kidney, where it produces a chronic and often very stubborn inflammation. In the bladder this is called *cystitis*; in the pelvis of the kidney, *pyelitis*.

Extensive studies of bacteria in the feces of infants with diarrhea indicate that many cases of infantile diarrhea are due to certain particular kinds of *Escherichia coli*. These pathogenic *E. coli* can be distinguished only by immunologic studies of their antigenic structure. Some of these strains of *E. coli* are designated as 026:B6, 0111:B4, 055:B5, 0119:B14, 0127:B8, 086:B7, and so on. The numbers and letters refer to antigens in the organisms. There are ten or more such strains known.

These organisms are particular nuisances in children's institutions and nurseries. They are spread about by hands and fomites, as are other enteric pathogens, and at times are very difficult to eradicate.

## PARACOLONS AS PATHOGENS

Some especially designated species or groups of the *Paracolobactrum* are regarded by some authorities as possible causes of diarrheal conditions in human beings, much as are certain specific types of *Escherichia coli*. Groups of *Paracolobactrum* possibly so implicated are the "Arizona group," the "Bethesda-Ballerup group," the "Providence group," the "32011 group," and so on. The Providence and Bethesda-Ballerup groups comprise mainly *P. intermedium*-like species, the 32011 group *P. aerogenoides* species.



Some of the *Paracolonobacterium* contain antigens found in *Salmonella*, *Escherichia* and *Shigella*. In fact, all of the genera in the whole grand family of Enterobacteriaceae are related more or less to one another by the possession of antigens common to one or another species.

#### THE PARVOBACTERIACEAE

The family Parvobacteriaceae ("little bacteria") was established to provide a convenient grouping for a number of species of true bacteria which are relatively small ( $0.3\mu$  by  $2\mu$ ), gram-negative, non-sporeforming, non-motile parasitic, mostly aerobic rods. As a group, they represent the highly evolved type of bacteria, requiring complex organic media for their optimum growth. In general they lack the synthetic versatility and the ruggedness necessary to growth and survival in the outer world, which characterize such organisms as the *Aerobacter* and *Pseudomonas*. No saprophytic species of any genus in this family is known. All appear to cause disease.

#### GENUS PASTEURELLA

Pasteurella are named for Pasteur who founded the science of immunology on his studies of vaccination against *Pasteurella avicida*, the cause of fowl cholera. *P. avicida* is now regarded as a variant of the type species of the genus, *Past. multocida*. Other variants of *P. multocida* have previously been named for animals: *P. bovicida* (cattle); *P. suilla* (swine); *P. muricida* (rats); etc. They differ only slightly in biochemical properties. They are highly pathogenic for most birds and animals; much less so for man. They cause tremendous losses to stock and poultry industries (*hemorrhagic septicemia*, "shipping fever"). The species causing bubonic plague in man is *P. pestis*.

THE MORPHOLOGY OF PASTEURELLA is rather distinctive. In pathological material the organisms are short, oval rods, about  $0.5\mu$  by  $3\mu$ , which tend to stain most heavily at the tips (bipolar staining). In cultures the bipolar appearance is often less definite.

SURVIVAL OF PASTEURELLA. *Pasteurella* succumb readily to heat and disinfectants, but resist drying in dust to some extent. For example, *Pasteurella multocida* can survive in, and is transmitted by, infectious dust, fomites and animal secretions from stables, railroad cars, stockyards, etc. The organisms invade the lymphatic system and blood stream and may easily be cultivated on infusion media from all of the organs and body fluids of infected animals. There are many small hemorrhages on various internal mucous surfaces, in the skin, and in the internal organs, hence the name "hemorrhagic septicemia." In the animal disease, there is much exudation of fluid from nose, mouth, eyes, etc. This fluid is highly infectious and transmits the disease in flock, barnyard and stable. The disease is rapidly fatal as a rule.

CONTROL. These epizootics can be controlled only by rigid isolation and slaughter of sick animals followed by prompt incineration or burial, and disinfection of the premises. There are bacterins which are of some immunizing value. Shipping fever may be reduced by passive immunization with serum, given just before shipping.

**Pasteurella Pestis.** From the standpoint of human disease the most important member of the genus is *Past. pestis*, the cause of bubonic and pneumonic plague in man. Morphologically and culturally *P. pestis* resembles

*P. multocida* but is slower and less vigorous in its growth and somewhat less active biochemically. In general, these organisms attack no proteins and few carbohydrates beyond glucose.

**BUBONIC PLAGUE.** This malady is a classical example of a bacterial disease transmitted by the bite of an insect. It is primarily a disease of rodents. It is conveyed to human beings by the bite of infected fleas, most important of which, in this respect, are *Xenopsylla cheopis* and *Ceratophyllus fasciatus*, the rat fleas. The fleas usually derive the plague bacilli from the blood of infected rats: *Mus norvegicus* (sewer rat) and *Mus rattus* (house rat). When the rats are infected they show lesions similar to those found in man. Rats and rat fleas maintain plague as an epizootic disease much like hemorrhagic septicemia among themselves for long periods and act, therefore, as an *animal reservoir* of plague bacilli. When rats become excessively prevalent in any community, human plague is apt to occur because the opportunity for rat fleas to bite human beings greatly increases. Crowded populations living near dumps or in dirty, unsanitary conditions suffer most. Conditions following the devastation of war, with breakdown of disease-control systems, are ideal for the development of rats and, therefore, of rat-borne diseases. The pages of history are filled with disasters to armies and civil populations attacked by plague. Daniel Defoe's "Journal of the Plague Year" and Winsor's "Forever Amber" give dramatic descriptions of the plague in London, 1665. The rats often die in great numbers from the disease and the fleas tend to leave the cooling bodies, jumping on to the first warm animal which passes. Dead or dying rats are, therefore, potentially dangerous.

In 1900 plague was first found in human beings in this country. It has since been found in rats and in rodents other than rats, especially ground squirrels or "prairie dogs." Many human cases have been traced to contact with wild rodents. The disease in woodland or wild-living creatures is often spoken of as *sylvatic* (forest) or *campestral* (prairies) plague. The control of plague in wild rodents, etc., is a field problem of great importance.

**Pasteurella Tularensis.** This organism much resembles *P. pestis* and *P. multocida* but is somewhat more exacting in its nutritional requirements. Infusion media with blood or serum are used, but little or no growth occurs unless the amino acid, cystine, or (what amounts to the same thing) some compound containing the sulfhydryl (-SH) group is added.

*P. tularensis* is found in much the same ecological relationship to rodents (rabbits, gophers, mice), biting insects (wood, dog and rabbit ticks; rabbit lice; deer flies; horse flies) and man as are *P. pestis*, fleas, and rodents. *P. tularensis* causes a disease called *tularemia*. (The name is derived from the Tulare swamps in California where early observations were made on this disease.)

**TULAREMIA ("RABBIT FEVER").** This is a plague-like disease in many American rodents and other wild animals. As in plague and hemorrhagic septicemia, there are enlargements of lymph nodes, swelling of the spleen, and the appearance of tubercle-like nodules in spleen, liver and elsewhere. The bacilli invade the blood stream from these foci just as *P. pestis* does in plague.

One common means of transmission of tularemia is through the handling of infected wild rabbits, as in the marketing of these animals for food and

pelts, hence "rabbit fever." In some sections of the country the disease is known as "Deer fly fever," being transmitted largely by the deer fly, *Chrysops discalis*. In Arkansas and adjacent regions the disease is largely tick-borne. It causes enormous losses among sheep if they graze in areas where there is tick-infested undergrowth. *P. tularensis* has been isolated from forest streams. The water is apparently infected by the carcasses of infected wild animals dying in the stream or on its nearby banks.

The term *pasteurellosis* is properly applicable to infections with *Pasteurella*.

#### GENUS BRUCELLA

*Brucella melitensis* and two closely related species, *Br. abortus* and *Br. suis*, cause *Malta fever* or *undulant fever* in man, a disease common all over the world. The generic name of the causative organisms is derived from the discoverer, Bruce, a British scientist who first (1887) found the organism now called *Br. melitensis* on the island of Malta\* in the spleens of persons infected by the organisms in goats' milk.

*Br. abortus* was first known as the cause of abortion in farm animals, especially cattle. It was discovered by a Danish worker, Bang, in 1895 and is still often called Bang's bacillus and the disease in cattle, Bang's disease.

*Br. suis*, commonly found in swine, was first observed by Traum in the United States in 1914. Because of the frequent presence of these organisms in the blood and tissues of farm animals, persons in contact with them are likely to become infected. Thus meat packers, cattlemen, hog raisers, persons who drink un-Certified or unpasteurized milk, bacteriologists and veterinarians most frequently contact the disease.

The fact that the organisms discovered in 1887 in Malta, in 1895 in Denmark and in 1914 in America are all closely similar species of one genus was revealed in 1918 by Evans in the United States.

**Isolation.** Brucellas are very small rods without distinctive morphological features. They grow rather slowly on first isolation from the blood, milk or tissues of infected animals, or from the blood of man. They are best cultivated on slightly acid (pH 6.8) liver-infusion agar, or on tryptose or trypticase-soy agar or broth. Excellent examples of selective-type media based on antibiotics have been devised to facilitate cultivation of these organisms from contaminated materials such as feces and milk. Polymyxin B, penicillin, Actidione and crystal violet are the inhibitory agents, permitting *Brucella* to grow. *Brucella* can also be isolated from blood by injecting it into living chick embryos. *Brucella abortus* will grow at first only in an atmosphere containing about 10 per cent CO<sub>2</sub>. The three species are closely similar but may be distinguished by special tests.

**Survival and Distribution.** They can survive for considerable periods in dairy products, water, soil, dung, dust and meats, and are transmitted by these vectors. The tissues and fluids associated with an aborted animal are highly infectious.

The three species, though originally associated with certain animals, are not restricted to those animals but each may occur in any of the three species mentioned, as well as in man, dogs, horses, and possibly poultry.

\* Malta was called Melita by the ancients because of the fine honey (Latin = mel) found there; hence, Melit-ensis.

# GENERA *HEMOPHILUS*, *BORDETELLA*, *MORAXELLA*

Originally all of these organisms were included in the genus *Hemophilus*. This is named for the fact that the organisms were all thought to require blood\* for growth. It is now clear that some do not. These have been placed in the genera *Bordetella* and *Moraxella*. All are morphologically similar small rods though they are pleomorphic and often vary from coccoid to long, filamentous or distorted forms.

The genus *Hemophilus* includes *H. influenzae*† and several other pathogenic species, notably *H. ducreyi*, cause of the venereal disease, chancroid ("soft chancre").

The hemophils are excellent examples of the highly adapted and dependent bacterial parasite. These cannot live without certain blood components. One of these, long known as the "X factor," is the iron complex called *heme*, which is part of the red coloring matter of erythrocytes. Besides *heme*, *Hemophilus* require for growth a "V factor," now identified as coenzyme I,‡ found in erythrocytes.

**Bordetella.** This genus includes *B. pertussis*, the cause of whooping cough, discovered by a Belgian scientist, Bordet. On initial isolation from patients these organisms are truly and rigidly hemophilic, but on subculture soon become adapted to growth without either X or V factor. They do, however, require nicotinic acid (Niacin), a part of the coenzyme molecule.

*Moraxella* are very similar organisms which cause infectious conjunctivitis ("pink eye").

## SUMMARY OF ROD-SHAPED BACTERIA

Since all rods in this book except nitrogen bacteria (next chapter) have now been discussed, an inclusive list of rods is given at this point for the convenience of the student. This is *not* a *classification*. It is merely a systematized collection of data.

**Table 28.** *Rod-shaped Bacteria of the Sub-order Eubacteriineae Grouped on the Basis of 5 Principal Characteristics: Form, Sporulation, Gram-reaction, Motility, Flagellation.*§

### I. STRAIGHT RODS

#### A. Non-sporeforming

#### 1. gram-positive

#### a. Motile

- (1) Flagella, polar.....none known
- (2) Flagella, peritrichous.....*Listeria*

#### b. Non-motile

- (a).....Lactobacilleae
- (b).....Corynebacteriaceae  
(exc. motile *Listeria*)

\* *Hemo* is from the Greek for blood; *philus* from the Greek for liking or requiring.

† Formerly thought to cause influenza. Influenza is now known to be due to viruses.

‡ Phosphopyridine nucleotide. This is part of an important respiratory enzyme.

§ Exceptions occur in most groups. Some of the exceptions are listed. Others are unknown or are due to "spontaneous" variations and therefore unpredictable. The ill-defined and heterogeneous group listed in "Bergey's Manual" under Bacteriaceae is not included.

Table 28. Continued

## 2. Gram-negative

## a. Motile

## (1) Flagella, polar

- (a).....Pseudomonadaceae
- (b).....*Nitrosocystis*
- (c).....*Nitrosomonas*
- (d).....*Hydrogenomonas*
- (e).....*Methanomonas*
- (f).....*Thiobacillus*
- (g).....*Azotobacter*

## (2) Flagella, peritrichous

- (a).....Enterobacteriaceae  
(exc. non-motile *Shigella*  
and *Klebsiella*)
- (b).....Achromobacteriaceae  
(exc. 2 non-motile *Alcali-*  
*genes* sp. and 5 *Achromo-*  
*bacter* sp.)
- (c).....Rhizobiaceae

## b. Non-motile

- (a).....Parvobacteriaceae  
(exc. 2 or 3 motile  
*Bacteroides*)
- (b).....*Nitrobacter*
- (c).....*Shigella*

## B. Sporeforming

## 1. Gram-positive

## a. Motile

- (1) Flagella, polar.....none known

## (2) Flagella, peritrichous

- (a) (generally strictly aerobic)..*Bacillus*  
(exc. non-motile *B. an-*  
*thraxis* and 3 or 4 others)
- (b) (generally strictly  
anaerobic).....*Clostridium* (exc. non-  
motile *Cl. perfringens* and  
7 others)

## II. SPIRALLY CURVED RODS

## A. Non-sporeforming

- 1. Gram-positive.....none known

## 2. Gram-negative

## a. Motile

## (1) Flagella, polar

- (a).....*Vibrio*
- (b).....*Cellvibrio*
- (c).....*Cellfalcicula*
- (d).....*Thiospira*
- (e).....*Spirillum*

## (2) Flagella, peritrichous.....none known

## b. Non-motile.....none known

## B. Sporeforming

- 1. Gram-positive.....none known

## 2. Gram-negative

## a. Motile

## (1) Flagella, polar

- (a).....*Desulfovibrio* (and/or  
*Sporovibrio*)

## REFERENCES

- Dack, G. M.: Symposium on methodology for *Salmonella* in food. Bact. Rev., 1955, 19:275.
- Editorial: Some important lessons from the Lancaster, Pennsylvania, paratyphoid fever epidemic. J.A.M.A., 1956, 46:345.
- Edwards, P. R., and Ewing, W. H.: Identification of Enterobacteriaceae. Burgess Publ. Co., Minneapolis 15, Minn., 1955.
- Ewing, W. H., Tatum, H. W., Davis, B. R., and Reavis, R. W.: Studies on the Serology of the Escherichia coli group. Monogr. of the Communicable Disease Center, U. S. Pub. Health Service, Atlanta, Ga., 1956.
- Girard, G.: Plague. Ann. Rev. Microbiol., 1955, 9:253.
- Hagan, W. A., and Bruner, D. W.: The Infectious Diseases of Domestic Animals. 2nd ed. The Comstock Publ. Co., Ithaca, N. Y., 1952.
- Hardy, A. V., and Galton, M. M.: Salmonellosis. Am. J. Trop. Med., 1955, 4:716.
- Hirst, L. F.: The Conquest of Plague. Oxford University Press, New York, 1953.
- Hughes, K. E. A.: The epidemiology of Salmonella infections in man. Roy. Soc. Health J. (London), 1957, 77:1.
- Jebb, W. H. H., and Tomlinson, A. H.: The nutritional requirements of *Haemophilus pertussis*. J. Gen. Microbiol., 1955, 13: 1.
- Jellison, W. L., and Kohls, G. M.: Tularemia in Sheep and in Sheep Industry Workers. Public Health Monogr. No. 28, 1955. Sup't of Doc., Washington 25, D. C.
- Kendrick, P. L., Nadolski, S. B., Eldering, G., and Baker, J.: Antigenic relationships of *H. pertussis*, the paraptussis bacillus and *Br. bronchisepticus* as shown by cross protection tests in mice. J. Bact., 1953, 66:166.
- Link, V. B.: A History of Plague in the United States, 1955. Public Health Monogr. No. 26. Sup't of Doc., Washington 25, D. C.
- Murray, R. G. E., and Truant, J. P.: The morphology, cell structure and taxonomic affinities of the Moraxella. J. Bact., 1954, 67:13.
- Neter, E., and 54 others: Epidemic and endemic diarrheal diseases of the infant. Ann. N. Y. Acad. Sci., 1956, 66(Art. 1):3.
- Pollitzer, R.: Plague. World Health Organization Mono. Series No. 22. Columbia Univ. Press, New York, 1954.
- Rowatt, E.: Amino acid metabolism in the genus Bordetella. J. Gen. Microbiol., 1955, 13:552.
- Shipping Fever of Cattle-Hemorrhagic Septicemia. Farmer's Bulletin No. 1018, 1953. Sup't of Doc., Washington 25, D. C.
- Spink, W. W.: The Nature of Brucellosis. Univ. of Minnesota Press, Minneapolis, 1956.

## The Soil as an Environment for Microorganisms

THERE ARE many different kinds of soils: heavy clays, desert sands, swamp muck, ocean beaches, Arctic tundra, forests, garden loam, and so on. Each presents a different environment for soil microorganisms. Each soil is a special study in itself. However, certain principles are applicable to many and we can consider some of these generalities in this chapter. We shall think principally about agricultural and other fertile soils as representative of familiar types.

**Composition of Soils.** Such soils are derived more or less directly from disintegration of rock; mainly by weathering (heat, cold, water, ice, wind, etc.) and biological action. The soil consists primarily of inorganic particles ranging in size from large boulders through gravel and sand to microscopic specks; mixed in varying proportions, all more or less compacted together, but having interstices between them due to their irregularity in shape. These interstices contain more or less water and/or air,  $\text{CO}_2$ ,  $\text{H}_2\text{S}$ ,  $\text{NH}_3$  and other gases in small amounts, the proportions of each depending on rainfall, drainage, winds, temperature, atmospheric humidity, microbial activity and other factors.

**Soil as a Culture Medium.** The water in good agricultural soil contains, in solution, ions like  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{S}^{--}$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{--}$ ,  $\text{CO}_3^{--}$ ,  $\text{PO}_4^{--}$ , and others, depending on the composition of the original rocks, on farm cropping, on manuring and fertilizing practices, on the microscopic and macroscopic flora and fauna, and on other factors. These ions, it will be seen, represent elements essential in culture media for all forms of life. In a fertile soil these elements in mineral form are supplemented by a variety of organic compounds derived from the decomposition of animal and plant residues: carbohydrates ranging in complexity from glucose to starches, cellulose and polysaccharide gums; nitrogenous compounds ranging from urea to peptones, amino acids and complex proteins; fats; waxes; organic acids like acetic; pyrimidines; vitamins; growth factors; and so on. Thus, the soil water is actually an excellent culture medium for many microorganisms.

**Variations in Soil.** The soil environment is a highly variable one. Obvious variables are daily and seasonal temperatures, and water content. It is clear

also that, if a heavy crop of clover and timothy grass is plowed under, the soil is aerated, some moisture is lost and an enormous amount of readily assimilable substances in the plant juices is introduced. These soluble substances (proteins, carbohydrates, lipids, minerals, vitamins, etc.) quickly undergo hydrolysis, metabolism and decomposition, with a consequent great increase in internal temperature, in acidity, in content of  $\text{CO}_2$ ,  $\text{NH}_3$  and ammonium salts, and in relatively simple organic food substances. These support a tremendous upsurge in numbers of all heterotrophic forms capable of thriving in such an actively fermenting, acid, partly aerobic, partly anaerobic environment.

### SOIL POPULATIONS

**Bacterial.** This new growth, under common conditions, could consist at first of most species of Enterobacteriaceae, Pseudomonadaceae, Bacillaceae and various protozoa; followed and superseded, as acidity increases, by the more acidophilic and aciduric Lactobacteriaceae, yeasts, molds and other fungi, and so on. The entire microflora and microfauna undergo repeatedly, rapid changes roughly comparable to those occurring in sewage decomposition but modified by the differences between the two types of environment. Later, the acids are metabolized or combined, carbonates, amines and ammonia are formed, and the acidity reverts to alkalinity, especially if the soil is well aerated by tillage and drainage. The tremendous growth of microorganisms temporarily depletes the soil water of its soluble compounds, especially those of nitrogen, phosphorus, potassium and sulfur, so that, for two weeks or more, crop plants find it a rather unfavorable medium. However, the microorganisms soon die and release the elements for crop use.

After the immediately available substances (soluble compounds and readily hydrolyzable solids) are decomposed, ammonia is changed to nitrates,  $\text{H}_2\text{S}$  to sulfates, numbers of microorganisms, both bacterial and protozoan, decrease; the types become stabilized. Dormant spores of spore-formers (molds, yeasts, fungi, Bacillaceae, Streptomycetaceae) replace the active vegetative forms; cyst-forms of protozoa replace the trophozoite stages. Finally, a more or less complete equilibrium is established, awaiting the next change; perhaps the planting of a corn crop with liberal application of lime and/or commercial fertilizer to stir things up once again.

**OTHER ORGANISMS IN THE SOIL.** It must not be thought that the bacteria mentioned above are the only or the most important organisms in soil. The "purifying" or scavenging activity of soil is equally dependent on numerous other forms of life. Prominent in this community are the Actinomycetales and the higher fungi which, as we have seen (Chapter 4), are active in hydrolyzing many resistant substances, including cellulose, chitin, keratin, lignin, and vulcanized rubber. Many of these actinomycetes produce antibiotics which suppress bacterial growth. *Syntrophism* is also evident.

The numerous protozoa, especially the flagellates, ciliates and amoebae, convert much organic matter into protoplasm. A principal item of their diet is bacteria, the basis of an important ecological "control" relationship in the soil as it is in sewage. Another control mechanism active in soil, water, and sewage consists of the phages.

Many worms, ranging from microscopic nematodes to large earth worms



("night crawlers"), the delight of our fishing enthusiasts, eat organic matter, digesting it and returning it to the soil in simpler, more soluble forms as food for plants. Similarly, larvae of insects such as Japanese beetles, and burrowing insects and animals all help to convert and transform organic matter in close relationship to the soil.

**The Soil a Microbial Universe.** In chapter 13 it was pointed out that many substances useful as food for microorganisms tend to be adsorbed upon surfaces immersed in fluid media. It was shown that a mass of tiny particles, like sand or charcoal in a fluid culture medium, furnishes multitudes of tiny protected niduses and extensive surfaces where digestive enzymes tend to be concentrated and foods to be abundant. Such favorable circumstances are found in moist, fertile soil. Each particle of soil has its film of moisture and its swarm of microorganisms on its surface. On a rainy day, in some angles and depressions, tiny pools or puddles may develop. In this fluid myriads of microorganisms grow.

The "top soil," to a depth of from a few inches to several feet, is indeed an entire universe where billions of minute creatures live their pigmy lives, multiply in their minute-long generation times, struggle together for space, food and survival and finally die, only to be replaced by others. Nevertheless, like human beings, however obscure, each leaves its effect.

#### SYNTROPISM IN THE SOIL

**Cellulose Decomposition.** The cellulolytic action of fungi (Ascomycetes, Fungi imperfecti, Basidiomycetes) and various actinomycetes has long been known. Cellulose decomposition is also caused by species of *Clostridium*, *Cellulomonas*, *Cellvibrio*, *Cytophaga*, *Bacteroides*, etc. Cellulose decomposition is often accompanied by an increase of temperature, as in manure piles, or in wet hay or straw. Thermoduric and thermophilic species are, therefore, active in such processes.

Cellulose is a polymer of glucose, being made up of many long molecular strands formed by the joining together of glucose units; just as a protein is built up by joining amino acid units. A train composed of many railway cars makes a passable analogy. When cellulose is hydrolyzed the glucose units are eventually all separated. Thus many microorganisms, while they cannot use cellulose at all, are able to thrive on the glucose produced when some cellulolytic organisms are active in their presence. This is a good example of soil syntropism.

Similarly, if proteolytic organisms digest animal or plant proteins in the soil, they liberate large amounts of soluble amino acids which can be used by numerous other species. The same is true when any complex or insoluble materials (fats, waxes, rubber, plastics, etc.) are hydrolyzed in soil or in sewage. The products are simple, soluble and support growth of many other forms. Usually cellulose, keratin (horn, hoof and hair), chitin, plastics and such materials are decomposed slowly by hydrolytic actions.

**Aerobe-Anaerobe Relationships.** Another syntropic or mutually helpful relationship is seen when, in some sequestered lacuna, perhaps only an inch below the surface of a well-aerated soil rich in organic matter, a strictly aerobic bacillus grows vigorously. It uses up all of the immediately-available, free oxygen. It thus creates a situation, locally, in which strictly anaerobic

*Clostridium* spores can germinate. The vegetative forms of this anaerobic species thrive for a time. Other strict anaerobes and facultative species can also thrive.

This relation of aerobe to anaerobe was often made use of in the laboratories of earlier bacteriologists to cultivate strict anaerobes. The two types (aerobe and anaerobe) were cultivated in two flasks connected by a tube, or in two separate culture vessels in a tightly closed jar.

**Formation of Humus.** The least digestible parts of plant tissues (cellulose, lignin,\* resins, etc.), and waxes, hair, horn, bone, etc., of animal carcasses, still undergoing slow decomposition, accumulate in the soil. The mixture of slowly decaying wood, bark, stems, leaves, roots and animal remains, make up a soft, spongy, brownish, residual material called *humus*. This is an extremely valuable constituent of soil. It improves the texture of soil, making it more friable; holds moisture like a sponge; and serves as a reserve store of organic food matter for microorganisms and crop plants. Woodland top soils and good "bottom lands" are particularly rich in humus. The plowing under of a heavy crop of clover, alfalfa, or grass ("green manures") or a heavy dressing of stable manure introduces into the soil the material for a good supply of humus.

#### BACTERIOLOGICAL EXAMINATION OF SOIL

Since fertile soil contains anaerobic organisms, strict aerobes, thermophils, autotrophs, heterotrophs, and other varieties having specialized growth requirements, no single method can be given for cultivating or enumerating soil bacteria in general. Several procedures are available for studies yielding approximate results.

**Plating Methods.** The plating method, as previously described, is applicable to the enumeration and isolation of bacteria in any substance, such as soil, water, milk, blood, feces, etc. Suitable modifications are made to meet the cultural requirements of the bacteria likely to be found in the substance examined.

**Selective Methods** are often used to cultivate or isolate particular species from the soil. The basic principle is like that underlying the isolation of coliform organisms from water by means of eosin-methylene blue medium or brilliant-green-lactose-bile broth. For example, a series of dilutions is placed in tubes or plates of medium containing cellulose as the only source of carbon. Only cellulose digesters can grow.

One may follow the clever scheme of Winogradsky to select and enumerate autotrophic oxidizers of ammonia (*Nitrosomonas* and related types). A medium is prepared with  $(\text{NH}_4)_2 \text{SO}_4$  and requisite minerals. It is solidified with silica gel.† The surface is coated with powdered lime ( $\text{CaCO}_3$ ) giving it a white, opaque appearance. As the  $\text{NH}_3$  [ $(\text{NH}_4)_2 \text{SO}_4$ ] is oxidized to  $\text{HNO}_2$  by the growing colonies of ammonia oxidizers, the  $\text{CaCO}_3$  is dissolved and a clear zone appears around such colonies.

\* Lignin is a woody substance of only partly known composition, in some ways resembling cellulose, but much harder, tougher and more resistant to hydrolysis. It occurs in roots and hearts of trees and woody plants.

† Since these organisms will not (in the laboratory) grow in contact with organic matter like agar or gelatin.

**Enrichment Methods.** These procedures all require cultural arrangements such that one sort of microorganism living in a mixed population is greatly favored while other sorts are discouraged. For example, to enrich Thiorhodaceae sea water is placed, with decomposing organic matter, in a closed bottle in the sunlight.  $H_2S$  is evolved, anaerobiosis and light favor photosynthesis, and so the sulfur bacteria can thrive, eventually outgrowing the other forms to a great extent.

**Microscopic Examination.** By making stained smears of soil and examining them with the microscope we may count various morphological types of bacteria and other microorganisms. The principal inaccuracy in this method is that dead as well as living cells are counted. Another arises from difficulty of staining some species and still another from errors in differentiating minute inert particles from bacteria, as well as morphologically similar species of bacteria from each other. By microscopic examination alone it has been found that bacteria inhabit only the upper layer of soil, seldom occurring in any large numbers below 2 feet from the surface. Good fertile loam may contain anywhere from 100,000 to 500,000,000 per gram. Among these soil bacteria we find every form of microbial morphology and almost every type of metabolism known.

## CYCLES OF THE ELEMENTS

### THE NITROGEN CYCLE

While the exceedingly important functions of organic hydrolysis, decomposition and humus production are carried out by a large and heterogeneous group of enzymically active and physiologically versatile soil microorganisms, certain highly specialized and very important activities are carried on by particular species having unique physiological properties. These specialized microorganisms are responsible for bringing into the organic scheme, by chemical combination, absolutely essential elements (nitrogen, sulfur, phosphorus, carbon, etc.) which otherwise would not become available as food for higher forms of life, including man. Under the influence of specific microorganisms these elements undergo cyclical alternations between the organic and inorganic forms; the reduced and oxidized forms; or soluble and insoluble forms. One of the best known of these alternations is called the nitrogen cycle.

**Processes in the Nitrogen Cycle.** This cycle comprises three main processes: I, nitrogen fixation;\* II, nitrogen oxidation; and III, nitrogen reduction (see Table 29). The nitrogen cycle is carried forward in the soil mainly by three types of bacteria: I, The nitrogen fixers; of which there are two sorts: A, non-symbiotic, notably the family *Azotobacteriaceae*; and B, symbiotic, genus *Rhizobium*.† II, The nitrogen oxidizing (nitrifying) bacteria, tribe Nitrobacteriae. III, The nitrogen reducing microorganisms, mainly heterotrophic saprophytes of many and various species.

\* Nitrogen fixation is the process of causing free nitrogen to combine chemically with other elements.

† *Rhizo* is from a Greek word meaning root; *bium* is from the Greek word bios, meaning life.

Table 29. Principal Organisms Involved in the Nitrogen Cycle.

- I. Nitrogen fixation:
  - A. Non-symbiotic
    - 1. Azotobacteriaceae  
*Azotobacter*  
*Beijerinckia*
    - 2. Miscellaneous others  
*Clostridium*, etc.
  - B. Symbiotic
    - 1. *Rhizobium meliloti*, *R. trifolii*, etc.
- II. Nitrogen oxidation:
  - A. Nitrobacteriaceae
    - 1.  $\text{NH}_3$  to  $\text{NO}_2$   
*Nitrosomonas*  
*Nitrosococcus*  
*Nitrosocystis*, etc.
    - 2.  $\text{NO}_2$  to  $\text{NO}_3$   
*Nitrobacter*
  - B. Miscellaneous others  
*Pseudomonas*  
*Aspergillus sp.*, etc.
- III. Nitrogen reduction (Ammonia production and denitrification)
  - A. Inorganic mechanisms  
Lightning
  - B. Organic mechanisms  
Various microorganisms causing ammonification and denitrification

### I. NITROGEN FIXATION

#### A. NON-SYMBIOTIC NITROGEN-FIXING ORGANISMS

In the atmosphere above 1 acre of soil it is estimated that there are some 35,000 tons of free nitrogen. Yet not a molecule of it can be used, as such, by higher plants, animals or man without the intervention of the nitrogen-cycle microorganisms. Nitrogen fixers combine atmospheric nitrogen with other elements to form organic compounds (in protoplasm) from which, upon their decomposition, the nitrogen is liberated in a form available to farm crops either directly or through further microbial action.

The first microorganism discovered (by Winogradsky, 1895) to possess the property of using atmospheric nitrogen in the synthesis of protoplasm directly and without symbiotic aid was *Clostridium pasteurianum*, common in boggy soils. Aerobic nitrogen-fixing bacteria (*Azotobacter*) were next discovered by Beijerinck (1901). Since those discoveries the phenomenon of non-symbiotic fixation of atmospheric nitrogen has been observed in numerous other *Clostridium* species, as well as in certain blue-green algae; many photosynthetic bacteria like *Rhodospirillum*; *Desulfovibrio*; many marine bacteria; bacteria (*Methanomonas*) that fatten on the waste gas ( $\text{CH}_4$ ) of the swamp-land cellulose digesters; and some soil fungi.

#### The Family Azotobacteriaceae

This family includes some of the most important non-symbiotic nitrogen fixers. The family consists of two main groups designated as genus *Azotobacter* and genus *Beijerinckia*, differentiated mainly on the basis of morphology, slime formation, pH range for growth and the fact that *Beijerinckia*

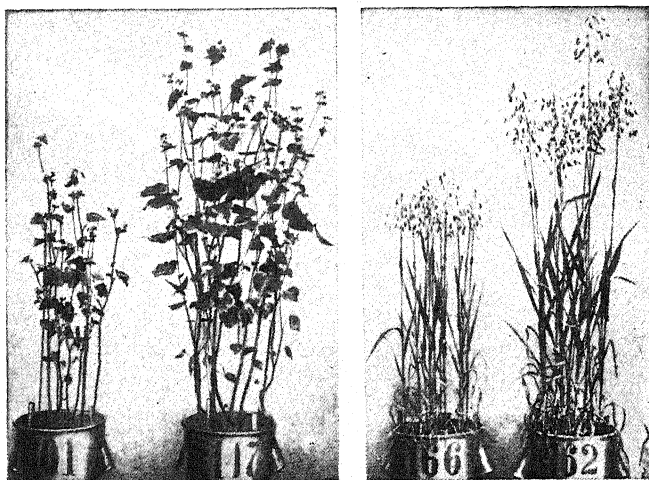


Fig. 40-1. Buckwheat and oats fertilized with sugar. Pots 101 and 166 were not treated; 117 and 162 received sugar. (From Löhnis and Fred, "Agricultural Bacteriology," McGraw-Hill Book Co., Inc., Publishers.)

seem to occur mainly in the tropics. Of the *Azotobacter*, *A. chroococcum*, *A. agile*, *A. beijerinckii* and *A. vinelandi* are recognized species; of the *Beijerinckia*, *B. indica* and *B. lacticogenes*.

**Genus *Azotobacter*.** The *Azotobacter* thrive especially in well-aerated, neutral or slightly alkaline (pH around 7.5), arable soils and are strict aerobes. They do not form spores. They are motile with numerous peritrichous flagella. They are large, usually rod-shaped bacteria, but quite pleomorphic and often yeast-like in appearance. They appear to multiply sometimes by budding. They require organic substance (various alcohols, organic acids, carbohydrates) as a source of energy and carbon but otherwise are like strict autotrophs and are retarded by the presence of organic matter such as peptone. Their enzymatic hydrolytic powers are feeble. They obtain their nitrogen directly from the air.

In the soil, the carbohydrates needed for their energy are probably derived ordinarily from the decomposition of cellulose. It has been found that carbohydrates added to the soil in the form of molasses, starch wastes and the like, act as fertilizer. The fertility results in part from the accumulation of nitrogen in the soil through the growth of the *Azotobacter* (Fig. 40-1). The nitrogen combined in their protoplasm is released on the death of the bacteria.

The *Azotobacter* grow readily in such nitrogen-free solutions as the following:

H <sub>2</sub> O.....	1000.0 ml
Mannitol (or other organic source of energy and carbon).....	15.0 gm
K <sub>2</sub> HPO <sub>4</sub> .....	0.2 gm
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.2 gm
CaCl <sub>2</sub> .....	0.02 gm
FeCl <sub>3</sub> (10 per cent aq. sol.).....	0.05 ml
Molybdenum salt.....	Trace

Adjust to pH 7.2. For solid medium, add 15 gm of agar before adjusting the pH.

The importance of molybdenum in this medium should be pointed out. In the absence of this element, even in minute amounts, nitrogen fixation will not occur. Molybdenum appears to activate an enzyme essential in the fixation process. It is a typical inorganic *growth factor*. It can be replaced by no other metal except vanadium. Magnesium is also essential to *Azotobacter* (and many other species). It activates the important, energy-transforming phosphorylating enzymes. *Azotobacter* require large amounts of phosphorus. They must have their sulfur as sulfate. A solution of this kind, when inoculated with soil, will support growth of *Azotobacter*, and they may be isolated from the fluid by streaking it on agar plates.

As *Azotobacter* grow they synthesize gummy polysaccharide substance about themselves in large amounts. They also secrete soluble nitrogen compounds into the medium around them; a valuable source of available N in the soil.

It has been shown that most species of *Azotobacter* will use other simple nitrogen compounds if available. However, *Azobacter* ordinarily have little choice in their nitrogen source and are restricted largely to atmospheric nitrogen.

## B. SYMBIOTIC NITROGEN-FIXING ORGANISMS

### *The Genus Rhizobium*

**The Rhizosphere.** There are several groups of microorganisms which live in very close association with the roots and subsoil tissues of growing plants, constituting what is generally called the *rhizosphere*, of which more later. Some of these microorganisms are dependent on plants for growth substances; some, like genera *Rhizobium* and *Nitrobacter*, produce valuable plant foods ( $-\text{NO}_3$ ), some are invasive and cause disease in plants; some invade but have established a symbiotic (or at least commensal) relationship with plants.

Here we shall observe the curious group of rhizosphere bacteria which are at once invaders, indispensable plant symbionts and fixers of atmospheric nitrogen: the genus *Rhizobium*.

**Genus *Rhizobium*.** When young and actively growing these bacteria are heterotrophic, aerobic, non-sporeforming, gram-negative rods, usually motile with variably placed flagella. Enzymatically they are restricted and feeble. They are pleomorphic, ranging in form from small, non-motile coccobacilli through larger cocci to flagellate "swarmers" and large, non-motile rods in a putative cyclical sequence. They grow on ordinary laboratory media, especially if made with yeast extracts. Their optimum temperature ranges around 20° C and pH around neutrality.

Their most characteristic activity and form are seen when they grow in the tissues of certain plant roots, where they live very well as "bacteroids."

**BACTEROIDS.** In smears made from crushed nodules from the roots of leguminous plants (beans, peas, clover, alfalfa, etc.) they are seen in the so-called *bacteroid* form: large, oddly angular, stellate or Y, V, T, X and L forms. These forms have metachromatic masses and bands which are thought by some to represent special reproductive mechanisms. Others regard these swollen forms as degenerative: the end of the supposed life cycle.

**NODULE FORMATION.** The *Rhizobium* are unable to hydrolyze cellulose

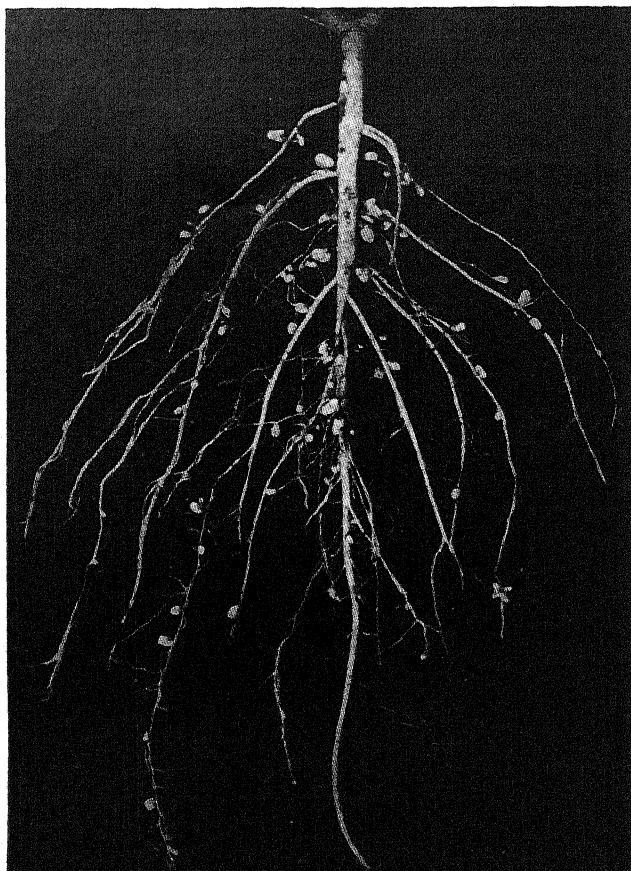


Fig. 40-2. Nodules on the roots of Alsike clover inhabited by nitrogen-fixing bacteria (Actual size.) (Swingle, "Plant Life," published by D. Van Nostrand Company, Inc.)

but find non-cellulosic points in the walls of cells at the tips of root hairs of legumes. Through these, entrance into the root tissue is made.

**✓INFECTION THREADS.** They utilize various carbohydrates, found in the soil or juices of the host plant to form characteristic gummy or mucilaginous, capsule-like coatings. These gummy coverings are of importance in helping them invade plant roots. As the rods advance, endwise, several abreast in their gummy coating, into the plant-tissue cells, the long, gummy thread is held within a hollow, cellulose-lined tube. The whole constitutes what is called an *infection thread*. These threads penetrate well into superficial root tissue. The bacteria enter the plant tissue cells and eventually assume the bacteroid stage. The presence of the bacteria stimulates multiplication of the plant cells around the localization, with resulting formation of a protective *nodule* (Fig. 40-2). Nutrients from the plant juices nourish the bacteria.

**Nitrogen Fixation.** The rhizobia, when thus growing in leguminous plant nodules, take nitrogen directly from the air. This is synthesized by them to compounds from which it is yielded to the plant in which they live. The

leguminous plants, when young, can utilize nitrates in the soil. If this is absent and the proper species of *Rhizobium* for nodule formation are not present in the soil, the plants cease to grow and die for lack of nitrogen (Fig. 40-3). They are dependent on the bacteria for their nitrogen.

**Soil Inoculation.** For this reason it is customary to inoculate virgin soils, or soils not known to support growth of legumes, with the proper species of *Rhizobium* preparatory to planting such crops as alfalfa or soy beans for the first time. State and federal Departments of Agriculture often make suitable cultures available to farmers. They are also available on the market under various trade names. The cultures are usually mixed with the seed before planting. It is important that the culture be fresh, and of a species suitable for the intended crop.

**SPECIES SPECIFICITY.** There are at least six species or varieties of the genus *Rhizobium*. They are: *R. leguminosarum*, associated especially with peas and vetches; *R. phaseoli*, infecting bean plants (genus *Phaseolus*); *R. trifolii*, common in red, white, crimson, and related clovers (genus *Trifolium*); *R. lupini*, found in lupins (genus *Lupinus*); *R. japonicum*, invading the soy bean (*Soja japonica*); and *R. meliloti*, which is used in planting alfalfa and sweet clover (genus *Melilotus*). They exhibit considerable species specificity. For example, *Rhizobium japonicum* produces nodules only on the soy bean, whereas *Rhizobium meliloti* will not do so. The latter will produce nodules only on species related to *Melilotus*, such as alfalfa (*Medicago sativa*). Strains of any given species vary greatly in respect to efficiency of nodule formation and nitrogen fixation, some being "good," and others "poor."



Fig. 40-3. Red clover; effect of nitrogen-fixing bacteria. No nitrogen was placed in the soil of either pot. The soil in the pot on the right was inoculated with *Rhizobium trifolii*. It is evident that the plant itself cannot fix nitrogen without the aid of the bacteria. (After Hopkins)



**VALUE OF NITROGEN FIXATION.** A nodulated crop like red clover, if well developed, may introduce as much as 100 lbs. of nitrogen per acre per season. In the form of a commercial fertilizer this would cost in the neighborhood of \$125.

Inoculation of swampy, acid soils is money wasted, as *Rhizobium* will survive and grow only in fertile, dry, aerated and nearly neutral soils. Crops of non-legumes grown in association with legumes (as vetch and rye or clover and corn) have for centuries been known to be superior. The legumes apparently release fixed nitrogen into the soil during growth. This nitrogen is used by the non-leguminous crop.

#### OTHER RHIZOBIACEAE

**Genus *Agrobacterium*.** This interesting and important group of soil bacteria comprises several species which, like *Rhizobium*, live in, or closely associated with, plant tissues. The genus consists of very small (0.2 by 3.0  $\mu$ ) non-sporeforming, gram-negative rods, motile with peritrichous flagella. They are facultative aerobes. They are not enzymically active. They are heterotrophic, growing well on ordinary laboratory media at pH around 6.8 and at temperatures around 25° to 39° C.

**PLANT TUMORS.** The type species, *A. tumefaciens*, is well known to the floral and horticultural industries as the cause of crown galls and tumors on plants such as the Paris daisy and many other families. Growth of this organism in the plant tissues stimulates local overgrowth (tumors) of the tissues. Studies of these effects have given some interesting leads in research on human neoplasms (cancer). A related species, *A. rubus*, infects only blackberries and raspberries. *Agrobacterium rhizogenes* acts in a similar manner but, as its name implies, it stimulates abnormal root growth especially, probably through the synthesis of a hormone-like factor (*auxin* or phytohormone). It causes "hairy-root" of pomaceous plants.

#### THE RHIZOSPHERE

The rhizosphere has already been mentioned as a zone of increased microbial growth and activity in the soil around the roots of plants. This mantle of microorganisms may be only in immediate contact with the roots or it may extend for inches around them. There are many interrelationships and interactions between plant roots and soil microorganisms. Some are favorable to plants, some indispensable, some unfavorable, others lethal. We know, for example, that some bacteria or fungi make nitrogen available to plants as nitrates or in organic form. Sulfur oxidizers make sulfur available as sulfates. Heterotrophic metabolism makes carbon available as CO<sub>2</sub> for photosynthesis. Production of acids by microbial action makes rock or bone phosphorus available as soluble phosphates. Some bacteria synthesize auxins or phytohormones (e.g., indole-acetic acid) which greatly stimulate root growth.

Plant roots reciprocate in kind. The roots of leguminous plants secrete soluble, organic, nitrogenous compounds into the soil around them to be used by microorganisms and other plants. Many plant roots appear also to give off simple, soluble, carbon compounds (foods for bacteria) like malic acid, pentoses, phosphatids, etc.

The sloughing off of bark and root coverings, as well as death of roots,

provides a rich source of carbohydrates and derivatives to support a luxuriant flora of nitrogen-fixers and other helpful forms close around the plant roots. The cellulose-digesters and other hydrolytic forms transform cellulosic plant material into humus and soluble foods for plants and microorganisms. A good heavy growth of microorganisms absorbs nitrogen, sulfur, phosphorus, potassium and other elements in soluble forms which might otherwise be removed (*leached*) from the soil by rain seepage and drainage. While the organisms withhold these elements temporarily from plant use (sometimes with damage to the plants) the elements are eventually released on death of the microorganisms. Thus, the higher plants act as a food manufacturer and storage-warehouse for microorganisms of the soil and rhizosphere, while the latter act as collectors, processors, and treasurers of foods for the higher plants.

**Antibiotics and Plant Diseases.** Among the unfavorable relationships between higher plants and soil organisms are (a) *parasitism* of plants by pathogenic microorganisms such as many species of *Phytophthora* (rots, wilts and spots), *Agrobacterium* (galls, hairy-root), fungi (rusts, rots, wilts), viruses (mosaics, "curly top"); and (b) *predation* by insects, rodents, nematodes, etc.

The organisms causing plant diseases live in the soil, often as saprophytes, but they possess pectinolytic\* enzymes and other properties enabling them to live in or upon plant tissues, causing disease.

Many plant pathogens are quite susceptible to antibiotics, including some of those used for treating infections in higher animals. We know that many of these antibiotics are produced by soil microorganisms: *Streptomyces*, *Penicillium*, *Bacillus* and numerous others. Scores of other antibiotics, not suitable for use in human or veterinary medicine, are excellent for control of soil pathogens and bacteria causing industrial spoilage (Chapter 44). It is scarcely to be doubted that the known microbial antibiotic-producers produce their antagonistic agents in their natural habitat and that they exert a tremendous influence on the soil microflora. They undoubtedly control plant pathogens to a great degree.

Antibiotics added to the soil in which plants are growing are soon found to be taken up in the plant and distributed to all its parts. Obviously, if a heavy growth of organisms which produce penicillin or streptomycin or polymyxin is present in the rhizosphere, not only is it going to prevent growth of many plant pathogens in the soil but, in addition, the plant is going to take the antibiotics into its structures and tissues and thus resist many plant infections. Most of the antibiotic producers, as we know (Chapter 20), are favored by carbohydrate-rich media. A moist, fertile soil rich in humus and organic matter likewise favors them and this, in turn, favors healthy crops.

There are many other microbial antagonisms among soil organisms which act to the benefit of the plant grower. For example, soil infested with *Phytophthora parasitica*, the fungal cause of "damping-off" of tomato seedlings, may be virtually rid of the pest by inoculation with *Penicillium patulum*. A species of *Trichoderma* (Chapter 4) produces a substance that greatly reduces infectivity of tobacco-mosaic virus. Many similar examples are found in the literature cited.

\* Pectin is a plant gum that holds the plant structures in place.

## II. NITROGEN OXIDATION

## TRIBE NITROBACTERIEAE

These, the nitrifying bacteria, are so called because they are concerned in *nitrification*, i.e., the oxidation of ammonia to nitrites, and nitrites to nitrates in the soil. This is an important phase in the nitrogen cycle since nitrates are one of the most expensive forms of plant food and are the most useful form (for many the only available form) of nitrogen for crop plants. The *Nitrobacterieae* are autotrophic and utilize ammonia or nitrites as sources of energy, the final product being nitrates. None forms spores. They are simple rods or cocci. Some are gram-positive, others gram-negative. The change from ammonia to nitrates is stepwise and involves two separate groups of organisms, each capable of carrying out only one of the two steps. The process may be outlined as follows:

1. **Ammonium Salts to  $\text{NaNO}_2$ .** (Genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosocystis* and *Nitrosogloea*.) The *Nitrosomonas*, et al., oxidize ammonia to nitrites, a process sometimes called *nitrosification*. The exact physiological mechanism of the oxidative process is not known. Calcium ions appear to be essential.

The *Nitrosomonas* are very small, oval rods with a single, polar flagellum. They are strictly aerobic, requiring abundant oxygen, and are very sensitive to acidity. Since oxidation of ammonia, and especially of ammonium sulfate, creates acidity due to  $\text{HNO}_2$  and  $\text{H}_2\text{SO}_4$ , they soon cease growth unless a soil is well limed or otherwise buffered. The optimum pH is around 8.6. They are strict autotrophs and can be cultivated only on silica gel or in other inorganic media. They are distributed in all arable soils.

*Nitrosomonas* can be cultivated in a solution of minerals like the following:

Ingredient	Per Cent
$(\text{NH}_4)_2\text{SO}_4$ (source of energy and nitrogen).....	0.20
$\text{K}_2\text{HPO}_4$ (buffer).....	0.10
$\text{MgSO}_4$ .....	0.05
$\text{FeSO}_4$ .....	0.04
$\text{NaCl}$ .....	0.04
$\text{CaCO}_3$ .....	0.10

2.  **$\text{NaNO}_2$  to  $\text{NaNO}_3$**  (Genera *Nitrobacter* and *Nitrocystis*). Most higher plants cannot utilize nitrites as their source of nitrogen. In fact, nitrites are toxic to many plants and some animals. The most immediately useful form of nitrogen for agricultural purposes is nitrate. Since nitrate does not occur spontaneously in soil, its development is dependent on the presence of the *Nitrobacter* et al., which oxidize nitrites to nitrates.

The *Nitrobacter* are non-motile rods. They occur in soil, rivers, streams, etc., and are world-wide in distribution. Under laboratory conditions they grow well only in the entire absence of organic matter. The *Nitrobacter* may be cultivated in solutions such as the preceding by substituting, as a source of energy, sodium nitrite for the ammonium sulfate.

**MISCELLANEOUS OTHERS.** In addition to the *Nitrobacterieae* certain heterotrophic bacteria have been shown to oxidize ammonia to nitrite:

*Pseudomonas*, (*Methanomonas*?), *Streptomyces*, *Nocardia* species. The second stage of nitrification ( $\text{NO}_2$  to  $\text{NO}_3$ ) appears to be carried out only by *Nitrobacter*, among the bacteria. However, species of soil fungus (*Aspergillus flavus*) appears to be capable of carrying out both steps; oxidizing organic nitrogen (possibly first forming  $\text{NH}_3$  from it?) to nitrite and nitrate.

### III. NITROGEN REDUCTION

**Importance of Nitrogen Reduction.** Were all combined nitrogen continuously to be used by living things and built up into protoplasm and to remain so, inextricably bound up as protein, then the agricultural use of manures, animal carcasses, fish, fertilizer, etc., would be of no avail. Were dead animals not to decay, manure not to rot, and dead fish to remain dead fish, the only forms of nitrogen eventually available would be the rare ammonia from lightning and free atmospheric nitrogen. Green plants and animals would have to await the slow activities of the nitrifying and nitrogen-fixing bacteria, or be limited to the use of the rare atmospheric ammonia or to ammonium salts, which are not favorable, in order to obtain properly combined nitrogen. Such, however, is not the case. As soon as protoplasm ceases to live, and as soon as any organic matter returns to the soil, it begins to undergo biological decomposition: *hydrolysis*, *putrefaction* and *fermentation*. Nitrites and nitrates undergo *denitrification*.

**Denitrification.** The reverse of nitrification is referred to as *denitrification*. In denitrification, nitrates are used by various facultative and anaerobic soil microorganisms as hydrogen acceptors and are reduced from nitrates to nitrites, gaseous nitrogen or ammonia, the extent of reduction depending on the species involved and the availability of free oxygen.

The reduction of nitrates accounts in part for the lack of fertility of constantly wet soils supporting growth of anaerobic species. Some of these species might be *Thiobacillus denitrificans* or various clostridia.

Thus we see that nitrogen, like sulfur, when related to the living world, is in a constant state of alternation between an oxidized state and a reduced state; an inorganic and an organic state. Its introduction from the inert atmospheric state into the cycle of life is in great part dependent on bacteria (Fig. 40-4).

With regard to the nitrogen of the decomposing organic matter, which alone concerns us for the moment, it is released from protoplasm and from animal and plant wastes chiefly through *ammonification*.

**Ammonification.** Protein is hydrolyzed to amino acids and these are broken down to other, simpler compounds when they are utilized by microorganisms. The amino groups ( $\text{NH}_2$ ) are split off in the form of ammonia ( $\text{NH}_3$ ). An ammoniacal odor is an outstanding impression in an uncleaned stable, or in the infant's diaper wet with urine, not promptly changed. The ammonia (which causes irritation and chafing of the infant skin) arises in great part from hydrolysis of urea ( $\text{O}=\text{C}\cdot\text{NH}_2\cdot\text{NH}_2$ ) by bacteria, among them *Micrococcus ureae*. The release of ammonia by the decomposition of organic nitrogen compounds is called *ammonification*. Note that the nitrogen is in its most reduced form.

Part of the fixed nitrogen represented by ammonia escapes into the atmosphere. Much is lost to the atmosphere from manure and compost heaps.



green and stable. Small amounts of volatile sulfur compounds ( $\text{H}_2\text{S}$ ,  $\text{SO}_2$ ) are washed into the soil from the atmosphere by rain.

**Reduction and Oxidation of Sulfur.** Partly and completely oxidized forms of sulfur, both inorganic forms like  $-\text{SO}_4$  and  $-\text{S}_2\text{O}_3$ , as well as organic forms represented by  $\text{R-SH}$ , are reduced by such organisms as *Desulfovibrio* and by many putrefactive heterotrophs such as *Clostridium* and *Proteus*, with the production mainly of  $\text{H}_2\text{S}$ . These reductive processes occur especially under anaerobic conditions, as in wet, heavy soils.

✓ Under aerobic conditions sulfur oxidizers like *Thiobacillus* and *Beggiatoa* and others oxidize the various reduced forms of sulfur to sulfates, in which form they are used by higher plants.

Thus we see sulfur in reduced forms oxidized by microorganisms as a source of energy to sulfate; sulfate is synthesized into reduced, organic forms by higher plants and subsequently by animals. From the organic sulfur compounds in the wastes and bodies of plants and animals sulfur is released by many heterotrophic saprophytes, largely as  $\text{H}_2\text{S}$ , which again furnishes a source of energy for sulfur-oxidizing microorganisms.

### THE CARBON CYCLE

Carbon, another essential element, undergoes similar cyclical transformations.

**Carbon Oxidation.** The more reduced forms of carbon ( $\text{CO}$ ,  $\text{C}$  and  $\text{CH}_4$ ) are oxidized in various ways: elemental carbon (mainly as coal) is oxidized (as fuel) to  $\text{CO}_2$ ;  $\text{CH}_4$ , comprising about 92 per cent of natural (petroleum) gas and of "sewer gas," is oxidized to  $\text{CO}_2$  by use as fuel; partly by such microorganisms as *Methanomonas*. Carbon in its partly reduced, organic forms and as  $\text{CO}$ , is oxidized to  $\text{CO}_2$  by combustion and by respiratory processes of plants and animals. Animal and plant wastes yield  $\text{CO}_2$  upon decomposition through fermentation.

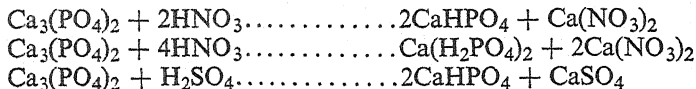
**Carbon Reduction.** Autotrophic microorganisms take carbon in its most oxidized form ( $\text{CO}_2$ ) from their environment and reduce it again, causing it to combine as organic matter; i. e., they "fix"  $\text{CO}_2$ . Green plants do likewise as part of the process of photosynthesis. Many heterotrophic microorganisms and probably some large animals also fix small amounts of  $\text{CO}_2$ , combining it as part of protoplasm. Anaerobic processes carried on in wet soils, sewage sludge, swamps, lake bottoms, and the like where cellulose is decomposed reduce carbon to carbon monoxide and methane.

So the carbon "goes round and round," alternating between organic and inorganic, reduced and oxidized.

### THE PHOSPHORUS CYCLE

The phosphorus cycle involves chiefly an alternation in form of phosphorus between the soluble and insoluble. In the soluble form it is used by, and exists in, living matter. It gains entrance to the soil in relatively insoluble forms in the phosphates of the rock from which the soil is derived. It is also added as  $\text{Ca}_3(\text{PO}_4)_2$  in the form of "bone meal" and in commercial fertilizers as "rock phosphates."

Phosphorus is liberated in part from its more insoluble compounds such as  $\text{Ca}_3(\text{PO}_4)_2$  by acids formed during nitrification and during oxidation of sulfur as follows:



Organic acids produced during fermentations in the soil and secreted by the roots of some plants also liberate soluble phosphates.

The soluble forms of phosphorus are apparently used as such by both higher plants and microorganisms. No phosphorus-oxidizing\* or reducing microorganisms are known hence there is no alternation between reduced and oxidized form as is the case with nitrogen, carbon and sulfur; only between organic and inorganic form; soluble and insoluble.

### MICROBIOLOGY OF PETROLEUM

No final conclusions as to the mode of origin of petroleum may be reached on the basis of present knowledge. However, it is generally held that it originated from living organisms and that bacteria had some part in it. Crude petroleum contains, in addition to various hydrocarbons (paraffine, kerosene, etc.) which are carbon-hydrogen compounds, compounds of nitrogen, sulfur, phosphorus and other elements in proportions and relations suggestive of derivation from organic matter. Studies of the subject make it pretty clear that: (a) the temperature of petroleum formation was within a range compatible with bacterial life ( $30^\circ$  to  $80^\circ$  C); (b) pressures up to 100,000 pounds per square inch or more are within the limit of microbial viability; (c) petroleum was formed in or near its present locations which, at the time, were probably sea bottom; (d) conditions were highly anaerobic; (e) salinities were not excessive. The great question is whether any known microorganisms, in any imaginable situations or under any conditions reproducible in the laboratory, could produce any higher homologues in the series than  $\text{CH}_4$  or  $\text{C}_2\text{H}_6(\text{C}_n\text{H}_{2n} + 2)$ . While some experimental evidence suggests that this *could* occur, no conclusive demonstrations on the point have been made.

All higher plants synthesize fats and carbohydrates. Deposits like the deposits which formed coal, when decomposed by certain microorganisms, could conceivably liberate large amounts of the hydrocarbons found in petroleum, but the exact mechanism is not clear.

**Destruction of Petroleum.** There is a large group of organisms which attack and destroy petroleum hydrocarbons. Two interesting species of these are in the genus *Methanomonas*: *M. aliphatica* and *M. aliphatica liquefaciens*. These oxidize higher homologues of methane: petroleum oils and paraffin.

Many other microorganisms can attack and decompose the hydrocarbons in gasoline, etc., and are of considerable importance in the petroleum industry as causes of spoilage. Among these are *Pseudomonas* and *Achromobacter* species, also *Alcaligenes*, *Mycobacterium*, *Aspergillus*, *Monilia*, *Sarcina*, etc.

\* As a source of energy.

**PROSPECTING FOR PETROLEUM.** The use of microorganisms like *Methanomonas* to find hidden sources of petroleum is of interest. Culture mixtures, complete in all respects *except carbon source*, are placed in flasks and inoculated with an appropriate species of organism able to utilize only petroleum vapors as carbon source. On being lowered into suspected oil-bearing strata and left for some days, growth will occur if hydrocarbon (petroleum) vapors are present. Patents have been issued for some processes of this kind. Difficulties arise from confusion of growth due to  $\text{CH}_4$  produced by cellulose-digesting anaerobic microorganisms of the surrounding soil, and hydrocarbon vapors from deep oil deposits.

## REFERENCES

- Anderson, G. R.: Nitrogen fixation by pseudomonas-like soil bacteria. *J. Bact.*, 1955, 70:129.
- Beerstecher, E.: *Petroleum Microbiology*. Elsevier Press, Houston, Texas, 1954.
- Bergersen, F. J.: The cytology of bacteroids from root nodules of subterranean clover (*Trifolium subterraneum* L.). *J. Gen. Microbiol.*, 1955, 13:411.
- Bryant, M. P., and Doetsch, R. N.: A study of actively cellulolytic rod-shaped bacteria of the bovine rumen. *J. Dairy Sci.*, 1954, 37:1176.
- Burkholder, P. R.: Cooperation and conflict among primitive organisms. *Am. Sci.*, 1952, 40:601.
- Davis, J. B., and Updegraff, D. M.: *Microbiology in the petroleum industry*. *Bact. Rev.*, 1954, 18:215.
- Elliott, C.: *A Manual of Plant Pathogens*. The Chronica Botanica Co., Waltham 54, Mass., 1951.
- Fisher, T., Fisher, E., and Appleman, M. D.: Nitrite production by heterotrophic bacteria. *J. Gen. Microbiol.*, 1956, 14:238.
- Harley, J. L.: Associations between microorganisms and higher plants (Mycorrhiza). *Ann. Rev. Microbiol.*, 1952, 6:367.
- Hungate, R. E.: The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.*, 1950, 14:1.
- Hutton, W. E., and ZoBell, C. E.: Production of nitrite from ammonia by methane oxidizing bacteria. *J. Bact.*, 1953, 65:216.
- Jensen, H. L.: The Azotobacteriaceae. *Bact. Rev.*, 1954, 18:195.
- Kamen, M. D.: Discoveries in Nitrogen Fixation. *Sci. Am.*, 1953, 188:38.
- Lochhead, A. G.: Soil microbiology. *Ann. Rev. Microbiol.*, 1952, 6:185.
- McBee, R. H.: The characteristics of *Clostridium thermocellum*. *J. Bact.*, 1954, 67:505.
- McElroy, W. D., and Glass, B.: *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, 1956.
- Odum, E. P.: *Fundamentals of Ecology*. W. B. Saunders Co., Philadelphia, 1953.
- Pine, M. J., and Barker, H. A.: Studies on the methane bacteria. *J. Bact.*, 1954, 68:589.
- Pratt, Y. T., Konetzka, W. S., Pelczar, M. J., and Martin, W. H.: Biological degradation of lignin. *V. Appl. Microbiol.*, 1953, 1:171.
- Reese, E. T.: Enzymatic hydrolysis of cellulose. *Appl. Microbiol.*, 1956, 4:39.
- Schmidt, E. L.: Nitrate formation by a soil fungus. *Science*, 1954, 119:187.
- Skinner, F. A.: Inhibition of the growth of fungi by *Streptomyces* spp. in relation to nutrient conditions. *J. Gen. Microbiol.*, 1956, 14:381.
- Stallings, J. H.: Soil produced antibiotics—plant disease and insect control. *Bact. Rev.*, 1954, 18:131.
- Stevenson, I. L.: Antibiotic activity of actinomycetes in soil as demonstrated by direct observation techniques. *J. Gen. Microbiol.*, 1956, 15:372.
- Thompson, L. M.: *Soils and Soil Fertility*. McGraw-Hill Book Co., Inc., New York, 1957.
- Van Schreven, D. A., Otzen, D., and Lindenberg, D. J.: On the production of legume inoculants in a mixture of peat and soil. *Antonie v. Leeuwenhoek J. Microbiol. and Serol.*, 1954, 20:33.
- Various Authors: *Plant Diseases*. Yearbook of Agriculture, Gov't Printing Office, Washington 25, D. C. 1953.
- Waksman, S. A.: *Soil Microbiology*. John Wiley and Sons, Inc., New York, 1952.



- Walker, J. C.: Plant Pathology. The McGraw-Hill Book Co., New York, 1950.
- Werkman, C. H., and Wilson, P. W.: Bacterial Physiology. Academic Press, New York, 1951.
- Wilson, P. W., and Burris, R. H.: Biological nitrogen fixation. *Ann. Rev. Microbiol.*, 1953, 7:415.
- Wise, L. E., and Jahn, E. C.: Wood Chemistry (part VI, Decomposition of Wood by Micro-organisms, W. G. Campbell). Reinhold Publishers, New York, N. Y., 1952.

## The Microbiology of the Atmosphere

NO MICROORGANISMS are indigenous to the atmosphere. Microorganisms of the air, like many of those in fresh surface water, are merely organisms of soil, decomposing materials, manure, etc., which have become attached to dust particles or droplets of moisture and are blown about by the wind. Microorganisms are more numerous in air in dry weather than just after a rain because rain washes them out of the air and "lays" the microbe-laden dust. Further, 50 to 60 per cent humidity is unfavorable to many microorganisms.

Obviously only species resistant to desiccation and exposure to sunlight can survive long in the air. Microorganisms commonly found in air are spores of bacilli like *Bacillus subtilis* and *Clostridium perfringens*, ascospores of yeasts, fragments of mycelium and conidia of molds and Streptomycetaceae, pollen, micrococci like *Sarcina lutea* and *Micrococcus luteus*, non-pathogenic corynebacteria (diphtheroids), chromogenic and non-chromogenic non-spore-forming rods like the coliform group, *Serratia marcescens*, *Chromobacterium* and *Flavobacterium*. In fact, almost any of the microorganisms discussed in this book except certain of the most fragile parasitic and aquatic species may at times be found in the atmosphere. Much depends on the location where air samples are examined, weather, speed and direction of air currents, population, etc.

**In the dust and air of theaters**, schools, hospital wards or the rooms of persons suffering from infectious diseases such organisms as tubercle bacilli, streptococci and pneumococci have been demonstrated, as well as other bacteria such as diphtheroids and staphylococci common in the normal mouth and throat. These are dispersed through the air in the droplets of saliva and mucus which are always produced by coughing, sneezing, talking and laughing (Fig. 25-5). Viruses of the respiratory tract are also transmitted by dust and air.

**The bacteria of the upper air** have been collected by means of aeroplanes or other aerial devices. For the collection of bacteria in the upper layers of the atmosphere, special apparatus must be used which excludes any possibility of contamination of the culture material, or other collection device, by dust from the airship or its occupants if such be used. Microorganisms are found in air even over the polar regions and far out at sea.

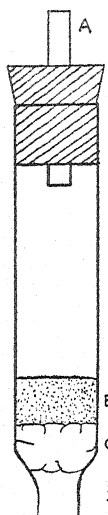


Fig. 41-1. Tube for collecting dust from the air for bacteriological analysis. Air enters at *A*, and deposits its dust on the sand (*B*), which is supported by a cotton plug (*C*). The air leaves at *D*. The sand is later washed with broth, from which a plate count is made.

More microorganisms are found in air over land masses than far at sea although, as we have seen, Darwin found various microbial spores a thousand miles at sea west of Africa (Chapter 33). Spores of fungi are more numerous than other forms, in both polar and tropical air masses, at altitudes of 8000 to 9000 feet. Total numbers of aerial organisms at such altitudes may range from less than 1 per cubic foot of air over oceans to several hundreds over land.

It is obvious that such information and such research is of enormous importance to agriculturists, since the spores of many pathogenic fungi causing very costly crop diseases can be transmitted from continent to continent by air currents. Many larger objects of biological importance are also carried long distances by high winds and air currents: fragments of soil with seeds and pathogens; parts of plants, often diseased; virus and bacteria-infected insects; birds; and even small rodents, fish, etc.

#### COLLECTION AND ENUMERATION OF AERIAL MICROORGANISMS

Bacteria in air may be collected by drawing the air through a tube containing a filter of wet sand or cotton (Fig. 41-1). There are several other general types of device for collecting microorganisms in the air; among them impingement devices, bubbling devices, and atomizing devices. In addition, it appears that the membrane filter (see Chapter 13) is adaptable to direct collection by filtration of air through the filter disk. Three representative devices are illustrated here. None collects and counts *all* of the microorganisms in the air sample tested although the membrane filter probably approaches closest to this objective (Figs. 41-2, 3 and 4).

#### DUST, DROPLETS AND DROPLET NUCLEI

Bacteria do not mount into the air unaided. Microorganisms found in the air are usually attached to particles of dust unless they happen to have come from the respiratory tract, when they are usually found coated with saliva or mucus. Even in this state they soon become attached to dust particles.

**Dust** in and around human dwellings usually arises from ash, soil, fabrics, bedding, clothing, carpets, soot, etc. The particles are usually relatively large ( $10\text{--}100\ \mu$ ) and tend to settle rapidly. Large (above about  $5\ \mu$ ) dust particles tend to be trapped in the upper respiratory tract and removed by mucus and saliva flow, sneezing, etc. Only the smallest (less than  $2\ \mu$ ) reach the lungs as a rule.

**Droplets**, as referred to the respiratory tract, are usually formed by sneezing, coughing and talking. They consist of particles of saliva and mucus. Each may contain hundreds of bacteria. Usually they are relatively large (of the magnitude of  $100\ \mu$ ) and, like dust, tend to settle rapidly in quiet air and to be trapped in the defensive hairs and mucus of the upper respiratory passages of persons inhaling the air containing them.

**Droplet nuclei** are the solid residua of such droplets after drying. Small droplets may, in a warm, dry atmosphere, dry before they reach the floor. They thus quickly become droplet nuclei. These are small ( $2\text{ to }5\ \mu$ ) and light and may float about for many minutes or even hours. Being very small, they tend to pass the mechanical traps of the upper respiratory tract and enter the lungs.

The size, weight, moisture content and opacity of air-borne particles are of

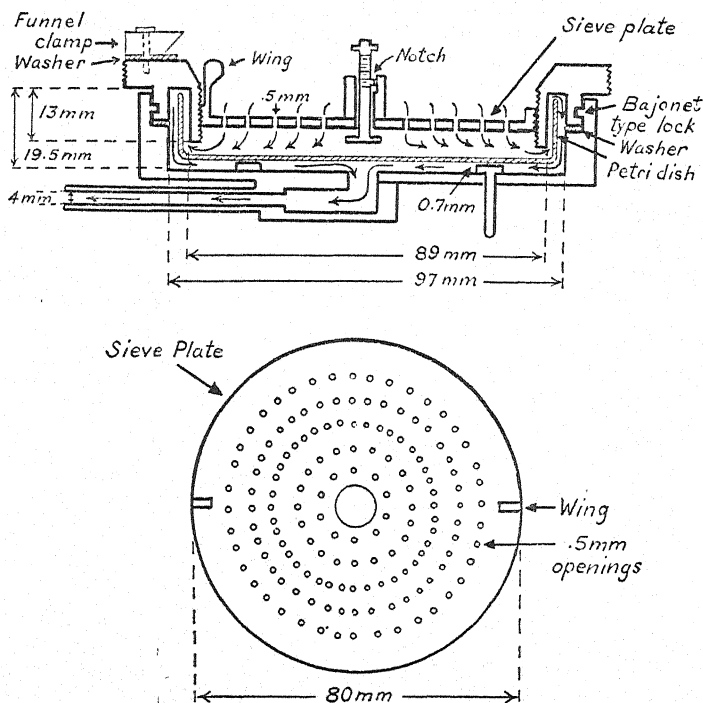


Fig. 41-2. Sieve device (a form of impingement device), with box and cover, containing a standard Petri dish with agar on which most of the particles of dust impinge. Arrows show the course of air through the perforations, along the agar surface, and out beneath the Petri dish. Sieve plate with wings is drawn separately. (duBuy, H. G., Hollaender, A., and Lackey, M. D., Supplement No. 184, U. S. Public Health Service.)

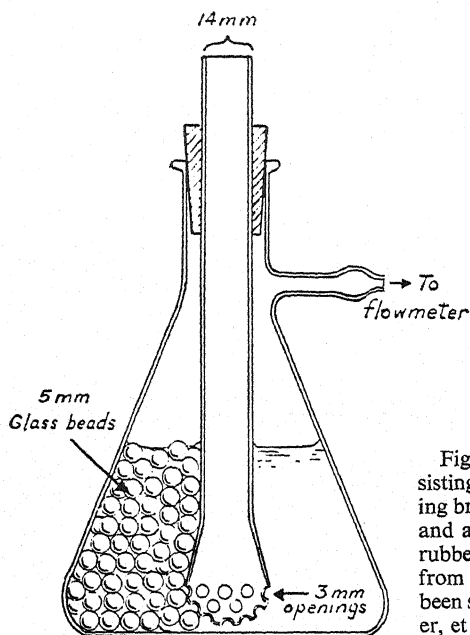


Fig. 41-3. Bead-bubbler device, consisting of a 250-ml suction flask containing broth, glass beads 5 mm in diameter, and a glass bubbler, kept in place by a rubber stopper. A plate count is made from the broth after sufficient air has been sampled. (Reproduced from Wheeler, et al., *Science*, vol. 94.)

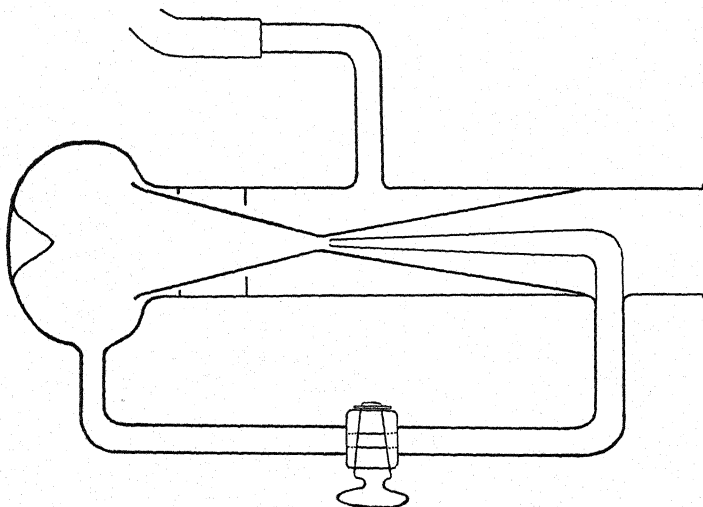


Fig. 41-4. Venturi-type air "scrubber," a form of atomizing device. Tube at top is connected to a vacuum pump. Air enters wide mouth at right. Passing at high speed through the narrowed throat at center, it draws fluid in a fine mist from the jet-tube opening. Passing into the bulb chamber at left the mist is diverted into circular currents by the papillate projection. The fluid portion condenses and is returned to the jet opening through the tube at bottom; the air is drawn upward through a system of baffles around the enlarged part of the air funnel and finally passes to the vacuum system. A large amount of air can thus be brought into contact with a small volume of fluid. An unmeasured proportion of the micro-organisms in the air may escape. A plate count is afterward made from the fluid.

great importance in considerations of methods of sampling air for bacteriological examination, and of methods for disinfecting air. For example, impingement methods will tend to catch larger and heavier particles, but not very small and light ones. Ultraviolet rays, sunlight, etc., may reach and kill organisms in droplets or nuclei if they are very small but opaque dust will protect microorganisms from ultraviolet irradiation which, as we have seen (Chapter 12), has little power to penetrate. Humid atmospheres (about 50%) have been shown to be more lethal than very dry or very moist atmospheres (20 or 80%).

### AIR POLLUTION

The crystalline, pure air our forefathers breathed is now subject to pollution from the industrial smokestack, the domestic chimney, and dust storms arising from uncropped land; to say nothing of liquors, garlic,\* tobacco, motor fumes, atomic explosions, radioactive fallout and flying machines (we might even include radio, television and political debate). Control over these involves engineering and other considerations foreign to this discussion. We may, however, consider the contribution of the individual human being to the all-enveloping pall of smog, perceptible or imperceptible, which now surrounds him in this day of advanced civilization from cradle to grave.

It is clear that the main source of microbial air pollution, so far as human sources are concerned, is the mixture of saliva and mucus from their upper respiratory tracts. Aside from methods of air sampling, already discussed, two important questions in studies of pollution of indoor atmospheres by human beings are: (1) what organism shall serve as an index of pollution by saliva and respiratory mucus? (compare use of *E. coli* and *Streptococcus faecalis* as index of sewage pollution, Chapter 37); and (2) what shall be used as a standard culture medium to isolate the organisms from the air? Final answers to these questions have not been formulated. As a test organism *Streptococcus salivarius*, a streptococcus of the alpha hemolytic type always(?) found in the normal(?) mouth, has been proposed. Objections to it are that it may occur elsewhere, and that it is a fragile organism which soon disappears from the air. In other words, does its presence truly indicate salivary pollution; does its absence prove the reverse?

Various media have been proposed for enumeration of bacteria in air. For general purposes the media used in milk or water examination are often used. As in all such enumerations, only those microorganisms capable of growth under the cultural conditions provided will be counted. For recovery of *S. salivarius*, special selective media have been devised. However, the field has not been fully explored and offers a good field for the ingenious and well-informed student.

**Control of Air-Borne Infection.** Thorough *dilution* of contaminated air by *ventilation* is a *very effective* means of controlling air-borne diseases indoors. However, it is sometimes expensive because of costs of heating or the installation of air ducts, blowers, etc. Under certain conditions disinfection or sterilization of the air is desirable. The problem has been given increasing attention. Three general methods in addition to ventilation are now available

\* The writer has nothing against garlic and often enjoys it on buttered toast.

for the control of microorganisms in the air. This does not take into consideration such procedures as passing air through mechanical filters, spray washing devices, etc. These reduce the numbers of organisms in air but do not disinfect or sterilize it.

**RADIATION.** A method widely discussed is the irradiation of air with *ultraviolet light* which, as pointed out in a previous chapter, is lethal to bacteria. A radiation of wave length 2537 Å is generally used as this is sufficiently bactericidal and at the same time not excessively irritating. Application of this is made in factories, storage warehouses, and some hospitals. Ultraviolet-producing electric lamps are attached to the walls, overhead or at other strategic points. Deflectors are used to prevent direct exposure to the rays, which can cause serious "sunburn," and to protect the eyes, which may be seriously and permanently injured by prolonged, direct observation of an ultraviolet source. A difficulty arises from the necessity that the microorganisms must circulate in air well above the heads of the people in the room in order to come within the range of action of the lethal rays. Only the lighter particles do this. Also, dust is little affected, and only places directly exposed are disinfected. Actually very bactericidal, ultraviolet irradiation is difficult of application except under special conditions: industry, laboratory, etc.

**BACTERICIDAL VAPORS.** Many substances are lethal to bacteria in the vapor phase, and several have been used for disinfection of air. Probably the most effective are propylene glycol and triethylene glycol. These are odorless, tasteless, nonirritating, nontoxic and not explosive or corrosive. They are highly effective in killing bacteria in the air as *vapor* although (curiously enough) relatively ineffective as concentrated aqueous solutions *in vitro*. It is the vapor molecules, and not droplets of fine mist of these substances that are the effective disinfecting agent. As little as 0.5 mg of propylene glycol in the vapor phase per liter of air can virtually sterilize heavily contaminated air in 15 seconds. Triethylene glycol is almost 100 times as effective.

Difficulties in the use of these vapors are chiefly of an engineering nature. Air conditioning appears inevitable to their effective use. The temperature and humidity of the air are important factors. If the air is cold and dry, or excessively humid, the effectiveness of aerosols is reduced. Relative humidities of around 40 per cent at about 76° C are favorable.

Other agents such as orthophenylphenol and related compounds have been recommended for surface application to supplement aerosols, especially as the phenylphenols cling to surfaces where dust settles and render them bactericidal for prolonged periods under ordinary atmospheric conditions. Creation of persistent films of disinfectant on floor and other surfaces is undoubtedly of importance in control of dust-borne disease (Chapter 18).

**DUST CONTROL.** In Chapter 25 it was pointed out that dust control is important in preventing disease transmission and the fact is re-emphasized here. Methods were described in the earlier discussion.

**Effectiveness of Methods.** Each of the methods mentioned above has been shown beyond doubt to be effective in reducing the *number of aerial bacteria*, both in experimentally contaminated laboratory atmospheres under various controlled conditions, and in such places as barracks, hospital wards, etc. Combinations of dust control by means of oil, and either vapors or irradiation, are even more effective. When it comes to reduction of *disease*, however, data

are less conclusive. The status of the problem is well summarized in two admirable reviews to which the reader desiring details is referred. A quotation from each is given here: "Conclusive evidence is not available at present that the air-borne mode of transmission of infection is predominant for any particular disease." [or that] "traditional methods of controlling contact infection [e. g., as by handshaking, kissing, eating utensils, etc.], can be ignored or relaxed." . . . "The available evidence strongly indicates that methods of air disinfection (ventilation, ultraviolet irradiation, and glycol vapors) are useful adjuvants to aseptic techniques in the reduction or elimination of air-borne infections in operating rooms and in contagious disease and pediatric wards."

"Admittedly, both ultraviolet light and glycols have their merits and demerits. Present knowledge is too limited to make any definite claims or predictions concerning their ability to reduce air-borne infection."

A great difficulty is that a person may spend his days in a protected environment such as an air-conditioned building with sterile air but that as he goes home in crowded subways or bus all of the expensive protection is nullified. This is well supported by published experience.

#### REFERENCES

- Army Epidemiological Board, Committee on Ventilation and Air Disinfection, Gordon M. Fair, Chairman: Present status of air disinfection. *Bull. of the U. S. Army Med. Dept.*, July, 1949, 9:542.
- Committee Report: Recent studies on disinfection of air in military establishments. *Am. J. Pub. Health*, 1947, 37:189.
- Committee Report: The present status of the control of air-borne infections. *Am. J. Pub. Health*, 1947, 37:13.
- duPuy, H. G., Hollaender, A., and Lackey, M. D.: A comparative study of sampling devices for air-borne microorganisms. *Pub. Health Rep'ts*, 1945, Suppl. 184.
- Gilcreas, F. W., and Read, H. R.: Bacteriologic studies in disinfection of air in large rural central schools. *Am. J. Pub. Health*, 1955, 45:767.
- Goetz, A.: Application of molecular filter membranes to the analysis of aerosols. *Am. J. Pub. Health*, 1953, 43:150.
- Kethley, T. W., Fincher, E. L., and Cown, W. B.: A system for the evaluation of aerial disinfectants. *Appl. Micr.*, 1956, 4:237.
- Klarman, E. G.: Surface disinfection and respiratory ills. *Modern Sanitation*, June, 1954.
- Krugman, S., and Ward, R.: Air sterilization in an infants' ward. Effect of triethylene glycol vapor and dust-suppressive measures on the respiratory cross infection rate. *J.A.M.A.*, 1951, 145:775.
- Lester, W., Jr., Kaye, S., Robertson, O. H., and Dunklin, E. W.: Factors of importance in the use of triethylene glycol vapor for aerial disinfection. *Am. J. Pub. Health*, 1950, 40:813.
- Loosli, C. G.: Problem of dust control for the disinfection of air. *Am. J. Pub. Health*, 1948, 38:409.
- Miller, W. R., et al.: Evaluation of ultraviolet radiation and dust control measures in control of respiratory disease at a naval training center. *J. Inf. Dis.*, 1948, 82:86.
- Moulton, F. R.: Aerobiology: a Symposium. *Am. Ass'n Adv. Sci. Publication No. 17*, Washington 25, D. C., 1942.
- Pady, S. M., and Kelly, C. D.: Numbers of fungi and bacteria in transatlantic air. *Science*, 1953, 117:607.
- Sonkin, L. S.: Application of the cascade impactor to studies of bacterial aerosols. *Amer. J. Hyg.*, 1950, 51:319.
- Williams, R. E. O., and Hirsch, A.: The detection of streptococci in air. *J. Hyg.*, 1950, 48:504.



## The Microbiology of Milk

MILK SECRETED into the udder of healthy cows is sterile. It has a pH of around 6.8. Some saprophytic bacteria of the outside environment are able to grow a short way up into the milk duct of the teat so that the first milk drawn usually contains variable numbers of them. This milk should be discarded. Such environmental saprophytes usually include *Micrococcus*, *Bacillus*, lactics, *Corynebacterium*, etc., from soil and dust. Except in cases where extra precautions are taken at the time of milking (Fig. 42-1) the milk receives its largest contributions of organisms from the pail and other dairy utensils, from soil and dust in the air, from the flanks, tail and udder of the cow, and from the hands of milkers.

**Types, Numbers and Significance of Bacteria Normally in Milk.** Ordinary market milk always contains strains of *Streptococcus lactis*, enterococci, non-pathogenic Enterobacteriaceae, as well as *Micrococcus*, Actinomycetales, species of *Pseudomonas*, *Propionibacterium*, *Chromobacterium*, *Lactobacillus*, *Bacillus*, *Clostridium*, yeasts, molds, etc. The presence of these non-pathogenic bacteria in milk is usually not a serious matter unless they cause the milk to sour quickly, putrefy, or develop undesirable flavors or conditions like "blue milk" (*Pseudomonas cyanogenes*), "red milk" (*Serratia marcescens*), "ropiness" (*Alcaligenes viscosus*) or when, because of their numbers, they show the milk to be stale or dirty. The development of many of these organisms may be prevented by clean handling (Figs. 42-2, 42-3); good, routine sanitization of milk handling equipment; prompt refrigeration; and by pasteurization, which consists in heating the milk to 145° F (62° C) for thirty minutes and immediately refrigerating.\*

**Changes in Flora of Milk.** Since milk is an excellent medium for bacterial growth, the numbers of bacteria in it increase steadily the longer it stands, especially if not pasteurized or refrigerated. Even if it is refrigerated, so that mesophilic growth is retarded, psychrophilic species may grow rapidly. Some of these can cause "ropiness," "off" flavors and other undesirable conditions.

\* "*Flash*" methods. Disinfection of milk is accomplished in many dairies by heating the milk rapidly in a tank, or in thin layers between metal plates, to 71.7° to 80° C and holding at that temperature for 15 to 60 seconds. These methods save time and are effective so far as sanitation of milk is concerned.

Common dairy psychrophils are *Pseudomonas*, *Achromobacter* and *Flavobacterium* species.

It is important that refrigeration be at very near 0° C. Much commercial refrigeration is at about 10° C, a temperature ideal for psychrophils. At best, such refrigeration is effective for not much over 24 hours.

Allowed to stand for several days at around 20° C the flora in raw market milk undergoes a series of changes which by now are familiar. Numbers of bacteria increase to almost astronomical figures. Enterobacteriaceae, yeasts

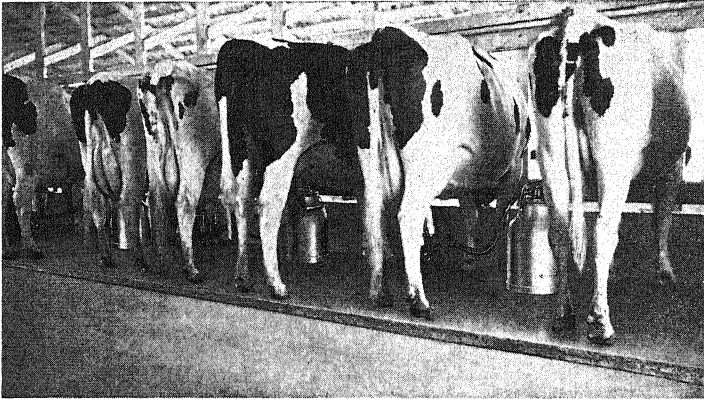


Fig. 42-1. Sanitary milk production. Note the cleanliness of the cows, the absence of dung and straw, the good lighting and ventilation and the milking machines. (Courtesy of the DeLaval Separator Company.)

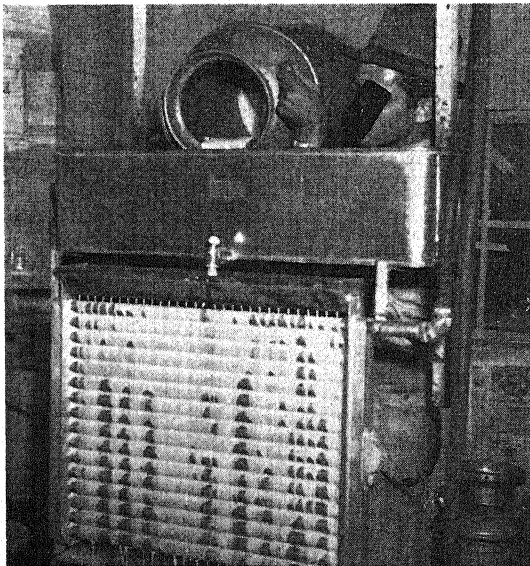


Fig. 42-2. Conditions contributing to high bacterial counts in milk. Milk cooler open to dust and dirt; not readily cleaned. (Photo courtesy of Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia.)

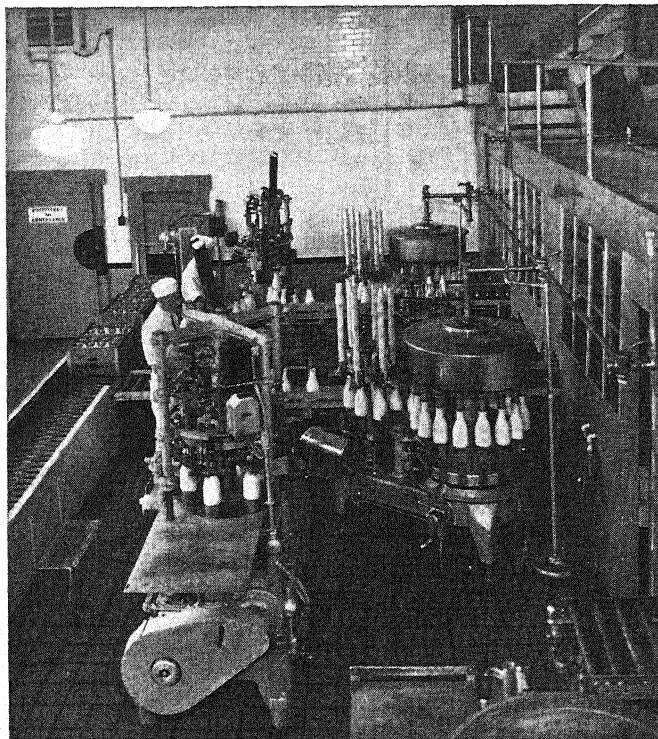


Fig. 42-3. Bottling milk in a modern, sanitary dairy. All of the piping can be demounted in a few minutes for steam sterilization and the floors and walls hosed down. In some modern plants light, transparent, plastic tubing is being used. The bottles in this picture have been steam sterilized just before filling. (Dodd's Alderney Dairy, Buffalo, N. Y. Courtesy of Cherry-Burrell Corp.)

such as *Torula cremoris*, sporeformers such as *Bacillus coagulans*, and other fermentative species which thrive best at a pH near neutrality, grow rapidly and dominate the picture at first. They ferment the lactose. As acidity increases they are inhibited. The aciduric lactic organisms then gain the ascendancy, especially *Streptococcus lactis*, *Leuconostoc*, and related organisms. When the acidity reaches a pH of about 4.7, curdling occurs. The curd shrinks and settles out. As the milk continues to stand, acidophilic organisms like *L. casei* tend to increase. Eventually, aciduric organisms capable of attacking lactic acid develop, especially aciduric fungi. These lower the acidity of the milk by destroying the acid and by production of alkaline products of protein decomposition: amines, ammonia, and the like. Since the carbohydrates (lactose) have been decomposed by this time fermentation does not reoccur.

Organisms capable of hydrolyzing the fat and casein now thrive: fungi, Bacillaceae, both aerobic and anaerobic, Pseudomonadaceae and many other saprophytes. As the oxygen content of the milk is diminished anaerobes gain the ascendancy and the odors and effects of "putrefaction" become evident. The casein is hydrolyzed; the milk is darkened. After the situation has some-

what stabilized a more prolonged decomposition continues, mainly by fungi and enzymes.

Pasteurized milk does not so promptly undergo souring because many of the fermentative species, being non-sporeformers and non-aciduric, are killed by the heat of the process. The milk may then undergo "sweet curdling" due to rennet formation by bacteria, especially aerobic sporeformers (*Bacillus*). Often it does not sour but the casein undergoes digestion and, later, putrefaction by the proteolytic enterococci, sporeformers and other heat-resistant, hydrolytically-active organisms.

**Significance of Coliform Organisms in Milk.** Coliform organisms are always present in market milk *before* pasteurization. They are present in hay, soil, dust, dung, utensils, etc. However, these organisms are killed if pasteurization is properly carried out. It is possible to determine whether there are any coliforms in milk by methods very similar to those used for determining coliforms in water (see Chapter 37). The details for all important laboratory procedures in connection with dairy products are to be found in "Standard Methods for the Examination of Dairy Products," 10th Ed., 1953, published by the American Public Health Association (see references).

Unless almost surgical precautions are used a few coliform organisms gain entrance to the milk *after* pasteurization, during cooling, bottling, etc. These do not harm per se but their numbers give a good indication as to post-pasteurization cleanliness and refrigeration or staleness. If considerable numbers of coliforms (more than about 1 to 5 per ml) are found it is evident that: (a) the milk has not been properly pasteurized; or that (b) it has been excessively contaminated by unclean conditions, possibly by sewage, feces or dung afterward; or that (c) the milk has been held unduly long above about 15° C after pasteurization.

**Factors Affecting Bacteria in Milk.** A factor of importance (especially to manufacturers of dairy products depending on early, rapid and luxuriant growth of certain bacteria, especially *S. lactis*) is the presence of bacteriophage lytic for that species. These 'phages are common in dust in and around dairies. They are known to interfere with many sorts of dairy work which is dependent on bacterial growth (see Chapter 44).

Another important factor to consider in bacteriological studies of milk is the possibility that the results have been influenced by (1) preservatives illegally added; (2) disinfectants from the dairy equipment; (3) antibiotics or other drugs used to control udder infections and otherwise administered to cattle. Coliform organisms are particularly undesirable in milk since they cause rapid souring, gassy fermentation and undesirable odors and flavors in cheese and other products made from the milk.

**The Phosphatase Test.** While the presence of excessive numbers of coliform bacteria in milk suggests staleness or improper pasteurization, more definite, rapid and accurate data can be obtained on this point by means of the *phosphatase test*.

This is a colorimetric determination based on the power of the heat-sensitive enzyme, *phosphatase*, normally present in fresh milk, to liberate phenol from phosphoric-phenyl esters. A standard amount of disodium-phenyl-phosphate, with some buffer ("buffer-substrate"), is added to 0.5 ml of milk to be tested. If the milk is unheated, the phosphatase normally present

in it will decompose the phenyl phosphate, liberating phenol. The phenol turns blue if a certain reagent (Folin-Ciocalteu phenol reagent) is added to the mixture. The intensity of blue color indicates the amount of free phenol present. When milk is heated at 143° F for thirty minutes (pasteurization), 96 per cent of the enzyme is destroyed. Heating above 145° F for thirty minutes insures complete inactivation of the phosphatase.

When milk has been underheated (in respect to either temperature or time) or when there is an admixture of raw milk afterward, the enzyme will be present in larger amounts than when the milk was properly processed.

The quantitative phosphatase test can be easily performed by any trained person and is a most valuable guide to the treatment which milk has received. This test will detect 0.5 per cent raw milk mixed with pasteurized milk, or 1 degree below standard temperature, or five minutes of underheating during pasteurization. Color values greater than that in a standard made by mixing 2.3  $\mu$ g of phenol per ml of boiled milk indicate progressive degrees of improper handling of milk.

**SIGNIFICANCE OF PHOSPHATASE TEST.** Over all ranges of temperature and times, *Mycobacterium tuberculosis* (the most resistant of the non-sporeforming infectious pathogens found in milk) is destroyed more quickly than phosphatase. Therefore, a heat treatment adequate to inactivate the enzyme likewise kills this organism and *all other common pathogenic bacteria*. A sample of milk that does not have more phosphatase present than the standard allows can be regarded as safely pasteurized and free from subsequent contamination with raw milk.

Certain bacteria, as *Lactobacillus enzymothermophilus*, can give falsely positive results in properly pasteurized milk because they produce phosphatase which remains active *even after proper pasteurization*.

**Counting the Bacteria in Milk.** In order to have some measure of the conditions under which milk has been produced and handled and to have a legal control over its sanitary quality, health departments and dealers have set up various standards by which to judge milk. Important among these standards is the number of bacteria present in milk.

**Enumeration of Bacteria in Milk.** **PLATE COUNT.** Numbers may be determined by one or more of several methods, legally-recognized procedures for which are detailed in literature cited. One of these methods is exactly analogous to the plate-count procedure described in Chapter 14.

**DIRECT COUNT.** Another is the direct microscopic examination of milk in a smear. This is prepared by spreading exactly 0.01 ml of the sample on a slide over an area of exactly 1 square cm. After staining, the smear is examined by means of a microscope calibrated so that the field is exactly 0.206 mm in diameter. The milk in this field represents 1/3000 cu. cm of milk. Using the data concerning the area covered and the volume of milk used, the total number of bacteria per ml of milk may be computed. Numerous large clumps of bacteria indicate unclean utensils. Many pus cells indicate infected udders. Streptococci indicate possible mastitis. It is common practice to report results in terms of clump counts. The method is quick and inexpensive. It is applied mainly to unpasteurized milk, since heating causes many of the bacteria to lose their staining properties.

**NUMERICAL RELATIONSHIP BETWEEN DIRECT AND PLATE COUNTS.** The

direct count is usually five to ten or more times as high as the plate count. This is because the former enumerates individuals, even those in clumps, and also dead bacteria. The latter enumerates only live bacteria capable of developing in the medium and at the temperature used. Each clump counts only as a single bacterium since each clump forms only one colony. Sometimes the clumps contain several dozen individual cells, yet a clump usually yields only one colony.

**The Reductase Test.** In the section on systematic studies of bacteria it was explained that actively growing bacteria bring about a lowered oxidation-reduction potential in their medium. The presence of bacteria capable of producing a low O-R potential can be detected by the use of methylene blue, for with a lowering of the O-R potential this dye becomes colorless. The test is used principally on raw milk and furnishes a rough approximation of the number and kinds of living bacteria present. In performing the test, 10 ml of milk sample are pipetted into a sterile tube and 1 ml of a standard methylene blue solution (final concentration 1:300,000) is added. The tube is closed with a rubber stopper and inverted 3 times to mix. It is placed at 35.5° C in the water bath immediately. At the end of each hour during the test it is inverted once. Observations are made after 30 minutes, 1 hour, and later.

The "*methylene blue reduction time*" is the interval between the placing of the tubes in the water bath and the disappearance of the blue color from the milk.

RESAZURIN, a dye related to methylene blue, undergoes a *series* of color changes depending on O-R potential changes, whereas methylene blue changes only from blue to colorless. Resazurin, therefore, permits readings of *degrees* of reduction at shorter intervals than does methylene blue. It is often used in place of methylene blue in the reductase test. An advantage of this is that one may estimate the amount of reduction that has taken place by noting (1) degree of color change after a definite time interval or (2) time required to produce a certain, stated color change. Thus, common procedures are the "one hour test" and the "pink-end-point test." Color standards are very exactly prescribed.

**Grades\* of Milk.** The actual numbers of bacteria permissible in milks of various grades vary in different cities. A good guide is the standard ordinance of the U. S. Public Health Service. Various localities may have somewhat different standards.

**GRADE A RAW MILK FOR PASTEURIZATION.** Grade A raw milk for pasteurization is raw milk from properly supervised producer dairies conforming to items of sanitation of workers, cattle, premises and equipment as prescribed in the ordinance. The bacterial plate count or the microscopic clump count of the milk, as delivered to the pasteurizing plant for pasteurization shall not exceed 200,000 per ml as determined by standard methods of the American Public Health Association or have a methylene-blue-reduction time of less than 5.5 hours or resazurin reduction time to a color designated as P7/4 of less than 2.75 hours.

**GRADE A PASTEURIZED MILK.** In all cases the milk shall show efficient pasteurization as evidenced by satisfactory phosphatase test, and at no time

\* Abstract of milk grades adapted from Pub. Health Bulletin No. 220 Milk Ordinance and Code of the U. S. P. H. S., (Revised, 1953; 3rd reprinting, 1956.).

after pasteurization and before delivery shall the milk have a bacterial plate count exceeding 30,000 per milliliter, or a coliform count exceeding 10 per milliliter, as determined by Standard Methods. The raw milk at no time between dumping and pasteurization shall have bacterial plate count or direct microscopic clump count exceeding 400,000 per milliliter, or a methylene-blue-reduction time of less than 4.75 hours or a resazurin reduction to color designated as P7/4 of less than 2.5 hours.

**GRADE B RAW MILK FOR PASTEURIZATION.** Grade B raw milk for pasteurization is raw milk which does not meet the bacterial standard for grade A raw milk for pasteurization, but which conforms with all other requirements. The bacterial plate count or the direct microscopic clump count of the milk, as delivered from the farms, shall not exceed 1,000,000 per milliliter, as determined by Standard Methods, or a methylene-blue-reduction time of less than 3.75 hours or a resazurin reduction time to color P7/4 of less than 2 hours.

**GRADE B PASTEURIZED MILK.** Grade B pasteurized milk is pasteurized milk which does not meet the bacterial-count standard for grade A pasteurized milk, and/or the provision of lip-cover caps of Item 20p, and/or the requirement that grade A raw milk for pasteurization be used, but which conforms with all other requirements for grade A pasteurized milk, and has been made from raw milk for pasteurization of not less than grade B quality, and has a bacterial plate count after pasteurization and before delivery not exceeding 50,000 per milliliter as determined by Standard Methods. Most communities now permit the sale of only grade A milk, pasteurized.

**Criteria of Good Milk.** The present status of microbiology of milk was well summarized by Robertson who said: "None of the routine laboratory procedures for estimating the number of bacteria in milk will determine whether or not infectious bacteria are present. The best assurance of freedom from infectious bacteria is that provided by proper pasteurization of the milk. The best assurance of pasteurization is that demonstrated by a satisfactory phosphatase test on the bottled pasteurized milk. The best assurance of freedom from recontamination in freshly bottled milk after pasteurization is a satisfactory coliform test in 1.0 ml portions of the bottled product."

**Certified Milk.** If milk is to be offered for sale unpasteurized, it is often required that it be produced only under very carefully supervised conditions. *The American Association of Medical Milk Commissions* has established rules and regulations concerning veterinary inspection of cows, medical inspection of personnel, sanitation of barns, utensils, etc. These very rigid regulations are often used by health departments and milk dealers in certifying qualified farms to produce such milk. It is usually called *Certified Milk* or "baby milk." The use of certified milk has much to recommend it, especially its cleanliness. It is said also to contain a larger proportion of certain vitamins essential for infants than milk which has been heated.

Most cities and states, as well as the A.A.M.M.C., require that all persons occupied in preparing certified milk, or, indeed, any food for the public, whether it be certified milk or not, be examined periodically for typhoid, paratyphoid, and dysentery bacilli. Examinations for *Corynebacterium diphtheriae*, tuberculosis, scarlet fever and other diseases are also required for certified milk handlers.



**Lactobacilli in Milk.** We have already made the acquaintance of these organisms (Chapter 35). They are important in the dairy industry in several ways. They are highly fermentative organisms, forming large amounts of lactic acid, and are aciduric and/or acidophilic. Some are slowly proteolytic and help to condition casein in the ripening of cheese. Several species are thermophilic and at least one species (*L. thermophilus*) grows well at temperatures as high as 62° C, a temperature quickly fatal to many non-sporeforming rods. As already noted this organism is a great nuisance in pasteurization plants. Lactobacilli are rarely associated with disease but a few cases of infection due to them have been reported.

**Fermented Milk Beverages.** In certain countries lactobacilli have been used for hundreds of years in combination with certain yeasts and streptococci, to produce beverages of fermented milk. The *yoghurt* of eastern central Europe, the *busa* of Turkestan, the *kefir* of the Cossacks, the *koumiss* of central Asia and the *leben* of Egypt are examples of these. In the old days, of course, the microbial nature of these processes was unknown.

**KEFIR.** In all of these fermented milks the lactobacilli act in company with other microorganisms (yeasts, lactic streptococci, various rods, etc.) of milk. For example, kefir, made from milk of various species of domestic animals, is prepared by putting "kefir grains" (small, cauliflower-like masses) into the milk. These grains consist of dried masses of lactobacilli, yeasts, *Streptococcus lactis* and probably other organisms held together in a matrix of coagulated casein. The kefir grains increase in size and break apart as the fermentation proceeds. The combined growth of the mixed flora yields a characteristically flavored, soured milk containing small amounts of alcohol. The kefir grains are found in the bottom of the vessels of fermented milk.

**YOGHURT.** The milk for yoghurt is commonly thickened by boiling or (commercially) by adding dried milk solids before the growth of the microorganisms. Various flavors, fruits, etc., are added. Yoghurt is nutritious and delicious!

**ACIDOPHILUS MILK.** In this country, *Lactobacillus acidophilus* is used in many commercial dairies and pharmaceutical houses to produce a soured milk product called "acidophilus milk."

**BUTTERMILK.** The product commonly sold in this country as "butter-milk" is in reality pasteurized skim or whole milk soured mainly by *Streptococcus lactis* with *S. citrovorus* and then beaten so as to produce a smooth, creamy beverage. It is a pleasant, nourishing drink. Addition of small amounts (0.15%) citric acid to the milk results in formation of increased flavor due to diacetyl produced by *S. citrovorus*.

**Milk as a Disease Vector.** There are two principal methods by which milk may become infectious for man and cause epidemics: I, the milk contains pathogenic microorganisms which are infecting the udder of the cow. The organisms of most importance in this respect are: (a) *Mycobacterium tuberculosis* var. *bovis*; (b) *Brucella*; (c) *Coxiella burneti* (the rickettsiae of Q fever); (d) *Streptococcus pyogenes*, Group A, introduced into the udder by a milker carrying the organisms. II, the milk contains pathogenic microorganisms which do not infect cows but are introduced into the milk, *after it is drawn*, by infected persons or other vectors. The more important organisms



of this sort are: (a) *Salmonella* or *Shigella*; (b) *Streptococcus pyogenes*, Group A; (c) *Corynebacterium diphtheriae*.

Ordinary pasteurization eliminates all except *C. burneti*. This requires a slightly higher temperature.

#### REFERENCES

- Brochure: Safe Milk: Its Importance to the Public Health. National Dairy Council, Chicago, Ill., 1950.
- Chalmers, C. H.: Bacteria in Relation to the Milk Supply. 4th ed. Edward Arnold, Ltd., London, 1955.
- Davis, J. G.: Laboratory Control of Dairy Plant. Dairy Industries Ltd., London, E. C. 4, England, 1956.
- Davis, J. G.: Milk Testing. Dairy Industries, Ltd., London, E. C. 4, England, 1955.
- Eckles, C. H., Combs, W. B., and Macy, H.: Milk and Milk Products. 4th ed. McGraw-Hill Book Co., New York, 1951.
- Facts about the Pasteurization of Milk, Leaflet No. 408, U. S. Dept. Agr., 1956. Sup't of Documents, Washington 25, D. C.
- Gainor, C., and Wegemer, D. E.: Studies on a psychrophilic bacterium causing ropiness in milk: I and II. Appl. Micr., 1954, 2:95.
- Grant, F. M.: Cleaning and Sanitizing Farm Milk Utensils. Farmers' Bulletin No. 2078. Sup't of Documents, Washington 25, D. C., 1955.
- Grant, F. M.: Farm Methods of Cooling Milk. Farmers' Bulletin No. 2079, 1955. Sup't of Documents, Washington 25, D. C.
- Greene, V. W., and Jezeski, J. J.: Influence of temperature on the development of several psychrophilic bacteria of dairy origin. Appl. Micr., 1954, 2:110.
- Hammer, B. W., and Babel, F. J.: Dairy Bacteriology. 4th ed. John Wiley and Sons, Inc., New York, 1957.
- Kaplan, A. S., and Melnick, J. L.: Effect of milk and other dairy products on the thermal inactivation of Cocksackie viruses. Am. J. Pub. Health, 1954, 44:1174.
- Lennette, E. H., Clark, W. H., Albimanti, M. M., et al.: Effect of pasteurization on *Coxiella burneti* in naturally infected milk. Am. J. Hyg., 1952, 45:227.
- Methods and Standards for the Production of Certified Milk. American Association of Medical Milk Commissions, Inc., 1256 Broadway, N. Y. City, 1957.
- Robertson, A. H.: Laboratory procedures in sanitary milk control. Am. J. Pub. Health, 1946, 36:1245.
- Standard Methods for the Examination of Dairy Products. 10th ed. Am. Pub. Health Assoc., New York, 1953.
- Steede, F. D. F., and Smith, H. W.: Staphylococcal food-poisoning due to infected cow's milk. British Med. J., 1954, ii:576.
- Tittsler, R. P., Pederson, C. S., Snell, E. E., Hendlin, D., and Niven, C. F., Jr.: Symposium on lactic acid bacteria. Bact. Rev., 1952, 16:227.
- Yale, M. W., and Kelly, C. D.: Thermophilic Bacteria in Milk Pasteurized by the High-Temperature, Short-Time Process. N. Y. State Agric. Exper. Stat. Bull. No. 630, Geneva, N. Y., 1933.

## The Microbiology of Foods

THE MODERN human dietary is broadly inclusive and embraces a wide variety of substances from many sources. It is subject to contamination and infection by many kinds of microorganisms and is treated by various processes involving many species of microorganisms; consequently the microbiology of foods is an exceedingly complex subject.

### DEFINITIONS AND CLASSIFICATIONS

We shall not concern ourselves here with a rigid definition of food but will include materials generally called food and most commonly used in the dietary of normal people in this country. Since water, milk and dairy products are dealt with elsewhere, they will not be included here. This leaves us to consider other common foods, which may for convenience in discussion be grouped as: (1) Fresh foods:\* meats, vegetables, fruits, fish, etc.; (2) the same foods preserved by (a) drying, (b) canning, (c) pickling or fermenting, (d) low temperatures, (e) disinfectants or bacteriostatic substances; (3) bread; (4) eggs.

Foods may be classified also on the basis of stability: (a) *perishable* foods such as meat, fish; (b) *semi-perishable* foods such as potatoes; (c) *stable* foods such as cereals, flour, sugar. Of course, any foods in this category which become wetted, overmoist or water-soaked are no longer stable.

We may divide microorganisms of foods into three general groups on a functional basis:

(1) those causing spoilage or undesirable changes in the food; (2) those producing desirable changes; and (3) those causing disease.

Regarding these microorganisms in each class of foods it is desirable to

\* For purposes of this discussion fresh foods may be defined as those recently harvested or prepared; which are in their natural or original state, not affected by any means of artificial preservation excepting refrigeration (not freezing) for limited periods (up to a week) and unchanged by effects of holding for sale or use beyond *slight* wilting or drying. Foods still edible but held so long that perceptibly undesirable changes in volume, weight, color, flavor, odor, appearance or other properties of fresh products have occurred may be classed as stale. An *accurate* definition of "fresh" and "stale" is very difficult indeed, as are chemical or bacteriological determinations of these conditions. We shall use the terms in their commonly accepted meanings.

know something of their source; their numbers; their effects on the foods; and how they may, if undesirable, be combated; if desirable, encouraged and fostered. Attempts to combat or encourage microorganisms in food are the basis of industries and research in this country involving hundreds of millions of dollars annually.

**Autolytic Enzymes.** In any discussion of the stability of foods, *autolysis* must be considered. When living cells die, certain little-understood enzymes act within a few hours or days to digest the cells. Autolysis proceeds best at optimal growth temperatures. Many meats undergo autolysis. Venison and poultry are often "hung" until tender; i. e., until some autolysis has taken place. Beef is more tender after a ripening period in refrigerators, partly due to autolysis. Autolysis may cause the uninitiated housewife to wonder why the pound of liver she bought in the butcher-shop seemed to melt away to a half-pound as she drove home on a warm summer day. Since conditions for autolysis and microbial growth are parallel to a great extent, autolysis under market conditions is usually accompanied by microbial action.

Leafy vegetables (lettuce, spinach, endive) are very apt to "spoil," due, in part, to autolysis. Bananas and other fruits are often made very soft during autolysis. Loss of weight, color, flavor and nutritive value result during excessive autolysis, and decomposition by yeasts, molds and bacteria can advance rapidly when over-autolysis has prepared the way. Hence, in preserving foods, efforts are made to prevent autolysis beyond a certain desirable "ripening" point, as well as to prevent decomposition by microorganisms. Usually the same preservative measures are equally effective against both. For example, in preparation of vegetables for preservation by freezing, autolytic enzymes, as well as some of the superficial bacteria, are partly destroyed by "blanching" or scalding for a few minutes before packing.

#### FRESH FOODS

**Meat.** When an animal dies of natural causes (disease, age, etc.), there is, before death, a short period (the agonal or moribund state) when there is a collapse of the defensive mechanisms which normally prevent invasion of the blood stream (and thence of the tissues) by microorganisms in the gastrointestinal tract, respiratory tract, skin and other body surfaces. Examination of muscle, liver and other tissues of such an animal, and especially lymph nodes which arrest bacteria coming from such sources, immediately after death reveals the presence, often in considerable numbers, of microorganisms characteristic of the intestine, respiratory tract, etc. These include Enterobacteriaceae, micrococci, fecal streptococci and respiratory streptococci; perhaps a few *Proteus*, *Pseudomonas*, and the like.

If a healthy animal is suddenly killed, as in the abattoir by a blow on the head, little post-mortem invasion of the blood and tissues occurs. Cutting a large vessel with a sharp, clean knife as in killing hogs, may introduce a few microorganisms, but not many. If the animal is then immediately dismembered in a cleanly manner, as is done in well-conducted abattoirs, relatively few, if any, organisms are to be found in the depth of solid tissues. This contributes greatly to the "keeping" quality of the meat, since, if the interior is sterile or nearly so, spoilage must proceed mainly on the surface and is

preventable by various treatments of the surface, such as curing or hanging in refrigerators under ultraviolet light.

**PRESERVATION BY ANTIBIOTICS.** Spoilage of fresh meat may be prevented for days and weeks by intravenous injection of antibiotics just before killing. Broad-spectrum antibiotics like chloramphenicol and the tetracycline group appear to give good results. The antibiotic is distributed throughout the tissues by the blood stream before the animal is killed. The method has proven of tremendous value in the tropics and in situations where refrigeration is not feasible. No perceptible change occurs in the meat for long periods.

An advantage of the method is that the meat need not be cooled immediately but can be kept warm so that, while remaining unspoiled, it undergoes, in 48 hours, the "tenderizing" (autolysis and other changes) usually achieved only by weeks of refrigerated storage, an expensive process. The amounts of antibiotic used are far below any toxic threshold and are largely or entirely destroyed by cooking.

Similar preservation, by the superficial application of antibiotics to dressed poultry in minimal amounts, was legalized for the poultry industry in 1955.

**USE OF RADIATIONS.** As pointed out in Chapter 18, exposure of microorganisms to certain electromagnetic radiations is lethal. Streams of electrons (cathode rays) under sufficient potential (1 million volts or more) can penetrate to a considerable extent yet not too much. When they impinge on a biological surface yet fail to penetrate, they liberate ions; i.e., they are ionizing radiations. Beta rays and gamma rays from radioactive cobalt and other wastes of atomic research have similar possibilities in this field.

Fresh, perishable foods in packages (plastic, paper, etc.) which can be readily penetrated by such rays can be sterilized *without heat*, and kept with *no refrigeration*. The passage of the microbicidal rays has, however, occasionally a perceptible effect on flavors and other properties of certain foods. The possibility of keeping foods fresh without refrigeration, whether by antibiotics or irradiation or other means, offers enormous advantages for ships, submarines, aerial travel; yes, even for space exploration!

**COMBINATIONS.** Various combinations of preservative agents are offering promising results. For example, acidity and heat together are enormously more effective than either alone. Pre-irradiation with gamma rays greatly increases the sensitivity of bacterial spores to heat. Other studies of this nature are greatly needed in this field.

**Microorganisms in Meat.** As already noted, a few organisms are found occasionally in the tissues and organs (especially lymph nodes) of fresh, healthy meat.

**THE SURFACE FLORA OF MEAT** depends obviously on the cleanliness with which it is handled and the length of time it is out of refrigeration. It becomes contaminated immediately it is exposed in the abattoir. Dust from hides and hair, bacteria on gloves, hands and cutting and handling instruments, all contribute to this. The principal damage done by such organisms is to cause putrefaction, decomposition of fat, and fermentation of carbohydrates in the surface tissues unless preventive and preservative measures are taken promptly. Some psychrophilic microorganisms cause unsightly but harmless blackening, greening, or other discoloration during refrigeration and may

produce undesirable tastes and odors such as rancidity due to decomposition of fats in the meats.

The organisms principally responsible for surface decomposition and spoilage of meat and other protein foods are bacteria of the genera *Pseudomonas*, *Bacillus* and *Micrococcus*, as well as various saprophytic Actinomycetales, yeasts and molds. Many other saprophytes may also be found, as *Achromobacter*, *Proteus*, enterococci, *Clostridium*, *Corynebacterium*, *Escherichia*, *Aerobacter*, etc. It is to be noted that these are all organisms of soil, dust, dung, etc.

Their numbers on the surface of the fresh meat may be estimated by direct microscopic counts of scrapings or of samples obtained by cutting sections of measured area from the surface of the meat with sterile instruments. Such cut samples are emulsified by shaking thoroughly or grinding in a measured volume of broth or saline solution. Dilution pour plates are made of the fluid and colony counts made as for water, soil or milk.

**Ground Meat.** The flora of freshly ground meat is commonly much richer in numbers and types of microorganisms than that of large pieces of meat like roasts because, in the ordinary processes of preparing ground meat, the meat is cut into small pieces, scraps are used and the surface contamination is thoroughly mixed with the meat as it is ground. It is also usually warmed somewhat. To this contamination is added the flora of the grinding machine, hands, implements, dust, etc. The microorganisms present, therefore, are those initially on the surface and in addition molds, yeasts, sporeformers, Actinomycetales and others from the environment. The nutritive conditions are ideal for a wide variety of fermentative and putrefactive organisms. Unless effectively refrigerated the bacteria grow throughout the whole mass and the meat spoils rapidly.

The admixture of antibiotics with the ground meat prevents growth of these spoilage organisms. The meat will then keep for many days at summer temperatures and for weeks in the refrigerator. *Bacteriological standards for ground meat* are difficult to evaluate. According to some workers using a medium like that used for enumerating bacteria in milk, incubated at 32° C, an aerobic plate count of 10 million organisms per gram of meat is a reasonable maximum. Occasional counts in ground meat of market quality may run into the billions per gram and this is exclusive of anaerobes, molds, and other organisms that do not grow under the cultural conditions provided. Unless the product is thoroughly refrigerated, or preservative antibiotics are used, decomposition sets in within a few hours at summer temperatures and the meat becomes putrid, rancid or otherwise offensive. The consumption of such meat is not necessarily dangerous but it may cause gastroenteritis.

**Bacteria in Comminuted Foods.** The same basic principles hold true for various "prepared" fresh products such as separated, fresh, crab meat; shucked shellfish; flaked, fresh fish; ground horseradish; chopped spinach; salad mixtures; cole-slaw, and the like. The more they are handled and the more they are chopped, ground or grated (comminuted) the more the surface microorganisms are mixed with the interior and the heavier the inoculation. Such foods are much more perishable than the same foods in solid masses, the interiors of which are not inoculated. They must be kept on ice at all times, and never sold if more than 24 to 48 hours old.

As in preservation of ground meats, admixture of antibiotics holds spoilage in check for many days, even without refrigeration. The acidity of products like slaw and horseradish, mixed with vinegar, tends to retard spoilage.

**Fish.** As is true of the flesh of mammals, the tissues of freshly killed, healthy fish are sterile or nearly so. Autolysis is slow in fish muscle. It is common practice on commercial fishing boats or landing places to "gut" fish immediately and pack them in ice. As in handling meat, the surfaces become contaminated with a variety of bacteria from sea water; the skin, scales and intestines of the fish; the hands and knives of the workers; the decks and cutting boards; and other environmental sources including ice and wash water in tanks, etc.

It is obvious that any contaminated products like fish, meat, milk, and the like are rich sources of nourishment for saprophytic bacteria, since they contain proteins, glucose, lipids, minerals, vitamins, organic phosphorus and sulfur and so on. The pH is near neutral. Such products will support a microbial flora, the extent of which will depend on cleanliness of environment, temperature of storage, salinity (in the case of fish), amount of handling, filleting, and other factors which influence spread and growth of environmental bacteria.

**INFLUENCE OF REFRIGERATION TEMPERATURES.** Failure or delay of refrigeration is one of the commonest causes of spoilage of any non-sterile food. Another is overlong holding at refrigerator temperatures several degrees *above* zero instead of *at or below* 0° C. Bacterial spoilage of many refrigerated foods (milk, fish, meats, vegetables, etc.) may occur twice as rapidly at around 5° C as at -1° C. Some psychrophils, especially marine forms important in spoilage of fish, grow at temperatures as low as -7° C.

In general, *growth* at low temperatures is not as rapid as at higher temperatures. However, *death* of the bacteria is retarded and maximum numbers of *live cells accumulate* at the lower temperature. When the refrigerated products are afterward gradually warmed for use, these enormous, active populations (along with their accumulated enzymes, slimes, pigments and other growth products) act very rapidly indeed to effect spoilage. (Compare with rapid cultivation methods, Chapter 17.)

Psychrophilic organisms commonly associated with spoilage of recently caught fish are species of *Micrococcus*, *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Corynebacterium* and *Mycoplasma*. *Clostridia* are also often present. Much depends on the waters from which the fish are taken, the species of fish and methods of handling. Anaerobes (*Clostridium*) which cause foul smelling decomposition apparently originate from feces of the fish and from sea-bottom mud.

Chlorinated water, ice with calcium chloride, and sodium nitrite have also been much used to preserve fresh fish. Admixture of antibiotics to the flaked ice in which the freshly caught fish are packed, or to minced fish, keeps the product in a perfectly fresh state, even unrefrigerated, for days on end.

**Oysters.** These and similar shellfish under market conditions are often rather heavily contaminated with dirt from the shells and from the benches at which "shucking" (removal of shells) is done. The flora includes marine forms similar to those on fish, organisms from soil, and dirt from equipment used in handling. If the water in which they are grown has a rich bacterial

flora, as is rather apt to be the case,\* the shell liquor may contain considerable numbers of various bacteria, including the coliform group.

Tests for the coliform group of bacteria in shell liquor, or the liquid around shucked oysters, are made in much the same manner as tests are made for this group in drinking water. The results are expressed in a similar manner. However, coliforms in oysters may be entirely non-sewage in origin, if the shell fish are taken from clean beds.

**Fruits and Vegetables.** The principles underlying the microbiology of fresh meat and fish products apply equally with regard to fresh vegetables and fruits. That is to say, the internal tissues of whole, healthy plants are free from bacteria but contamination of the surfaces by microorganisms from the soil, hands, packing machinery, packages, etc., can and does occur. The nature of this contamination obviously will be governed by its source and the mode of handling the product. Vegetables growing in the soil, such as root crops, have adhering to them soil saprophytes such as *Bacillus*, *Pseudomonas*, *Micrococcus*, *Clostridium*, *Lactobacillus*, enterococci, Actinomycetales, as well as species of cellulose- and pectin-digesters, yeasts, molds, soil protozoa and many others, depending on the nature of the soil: whether heavily manured, boggy, sandy, and the like. These organisms are usually held in check by drying, exposure of the surfaces of the vegetables to sunlight, and by refrigeration. *Intact* vegetable skins, free from superficial water, and not oozing juice from abrasions, resist invasion of microorganisms for considerable periods, especially if kept in a *cool, dry* place. Much depends on the kind of vegetables: whether they are bruised and scratched or have intact surfaces; are juicy or not; are acid or non-acid. Hard, non-succulent vegetables, such as turnips and potatoes, with whole skins, will stand storage better than soft, succulent, non-acid vegetables and fruits like lettuce, asparagus, spinach and ripe peaches, which offer good pabulum to microorganisms. These soon autolyze and then decay.

It seems not unlikely that spoilage of intact fruits and vegetables may be held in check by antibiotics, though this is still not completely developed.

**Eggs.** As soon as an egg is laid the outer surface becomes contaminated. If kept *cool* and *dry* the surface microorganisms cannot grow extensively. They are prevented from entering the egg for some days largely by the dried, mucilaginous coat, a sort of natural "varnish," on the surface. Various bacteria and molds can rapidly penetrate the shell if the mucilaginous coating of the shell is removed by washing, long storage, action of enzymes, or by mechanical means such as cleaning the egg with sandpaper. Such eggs do not keep well in storage.

Molds can grow on the outer mucinous coating if eggs are stored in humid atmospheres (above 70 per cent saturation) at ordinary climatic temperatures. These may give the eggs "off" odors and tastes as well as unsightly appearance and may penetrate the shell and contaminate the interior. They are frequently found in spoiled eggs. Eventually, at temperatures and humidity suitable for the growth of microorganisms, the mucinous protective film is decomposed

\* Oysters fatten best on sewage, and have long been known as vectors of typhoid fever if taken from polluted waters. Typhoid bacilli may live for two weeks or longer in live and shucked oysters. State and Government supervision of the beds has virtually removed infected oysters from the market. Formerly common, typhoid due to oysters is now very rare.

and the interior of the eggs is invaded with consequent decay, the rate depending on temperature and numbers and kinds of microorganisms.

The cleanliness of nests and birds, of hands, of containers and of storage places all affect the keeping quality of the eggs because these factors influence contamination of the shell. The principal sources of contamination are fecal matter brought to the nests on feet and feathers of the birds, and mud in wet weather, if they run in yards.

The flora of eggs, then, is largely of fecal and soil origin and includes the Enterobacteriaceae, Bacillaceae, *Pseudomonas*, *Micrococcus*, *Achromobacter*, *Corynebacterium* and the like as well as molds, yeasts and actinomycetes. This is very similar to the flora of dirty milk. The type of rot in eggs depends largely on the predominating organisms; usually a mixed flora is found. Most of the rot organisms are proteolytic and act anaerobically. The products of decomposition of eggs are, therefore, odoriferous. The protein is liquefied, the carbohydrates fermented, the fats hydrolyzed. The entire content of the egg becomes fluidified and darkened. A good deal of sulfur is present and much of it is reduced to  $H_2S$ , present under some pressure.

One of the common causes of spoilage of eggs stored at around  $15^{\circ}C$  is *Pseudomonas*. These penetrate the shells from soil, dirty wash water, etc. They impart an undesirable taste and odor to so-called "fresh" eggs long before overt, late-stage decomposition develops. It has been difficult to detect their early growth by any means. However, since many produce a fluorescent pigment (e.g., *Ps. fluorescens*, *Ps. ovalis*), they can readily be detected by the ingenious expedient of examining eggs in "black" (ultraviolet, 3200 Å to 4000 Å) light. Contaminated albumen is noticeably fluorescent through white but (unfortunately!) not brown shells.

**INFECTION OF EGGS.** Eggs may contain pathogenic bacteria when laid, even though the hen *seems* healthy. If the yolk is formed in an infected ovary, or albumen is laid onto the yolk in an infected oviduct, or if other parts of the egg are formed in infected organs, then it is not difficult to account for the presence of pathogenic bacteria in newly laid eggs. *Salmonella* species are commonly found.

**EGG PRODUCTS.** When eggs are broken under commercial conditions the contents are contaminated by the shell flora and microorganisms of the openers and containers. The bacteriology of commercial fresh egg products is (initially) largely that of the shells and of the environment. In commerce, the best (freshest) eggs are sold in the shell while mainly second or lower grade eggs are used for liquid, frozen or dried products. The flora of the latter products is therefore sometimes rather rich. Plate or direct microscopic counts of such egg products may run from one million to one billion or more per gram. This does not necessarily make the eggs unfit for use in cooking any more than high bacterial counts destroy the commercial value of lower grades of milk. If the egg products are not promptly frozen, refrigerated or dried, however, the heavy initial contamination quickly multiplies and ruins the product. One of the best means of avoiding high bacterial counts and consequent poor keeping quality in egg products is to handle only good, clean eggs, in clean machinery, in a clean environment. How far antibiotics can be effective in control of such spoilage remains to be determined.

**Bread.** The bakers' bread of the grocery store is produced by allowing



bakers' yeast (varieties of *Saccharomyces cerevisiae*) to ferment sugars (maltose, sucrose, etc.) in a mixture consisting mainly of flour and water, with some salt, sometimes softening and hygroscopic agents, and sometimes flavoring, raisins, caraway seeds, vitamins, etc., and shortening. Yeast (leaven) is mixed with water, flour, etc., to make dough. The dough is a plastic mass at first. Amylases and proteinases from the grains are present in flour and hydrolyze the starch and proteins. Sometimes malt\* is added to aid this process. Some proteins or *gluten* of the flour are partly hydrolyzed when the dough is made. The products of the hydrolysis support good growth of the yeast. Small amounts of cane sugar are often added in home bread making, both for flavor and to stimulate fermentation. Early growth of lactobacilli, derived from the grains, gives the dough an initial acidity which helps suppress undesirable organisms. The lactobacilli and other organisms also contribute to the flavor and aroma of the bread.

The yeast, or *leaven*, grows and produces  $\text{CO}_2$ ,  $\text{H}_2\text{O}$  and ethanol. The gas causes the bread to rise and gives it its foamy texture. Baking drives off the alcohol and partly dries the bread.

**LEAVENS.** An important phase of the work of some bakeries is the preparation of leavens. Many bakeries maintain their own leavens as trade secrets. The leaven may consist of a pure culture of a selected strain of yeast cultivated massively in aerated wort† made of malted grains or other starch derivatives.

Leaven may also consist of a mixture of pure cultures of *aerogenic* (gas-producing) bacteria: *Aerobacter cloacae*, *Lactobacillus brevis*, *Leuconostoc*, as well as yeasts. *Clostridium* species are undesirable in leaven because they produce rancid flavors. Bacterial leavens are used to prepare bread made of "sour" dough (*sauerteig*), and "self-rising" or salt-rising breads. In some bakeries, and the home, such leavens consist of previous lots of sour dough and are fortuitous mixtures. The most agreeably flavored sour-dough breads contain lactic acid and other products of bacterial fermentation, as well as diacetyl, a substance giving pleasant aroma to butter. These are produced by lactobacilli and by lactic streptococci, etc.

**MICROORGANISMS IN BREAD.** Most organisms in bread, aside from those added as leaven, appear to come originally from the flour or meal. Bacterial counts of flour range from a few hundred thousands to several millions per gram. These are mainly organisms of soil and fields: Enterobacteriaceae, *Lactobacillus*, *Micrococcus*, Bacillaceae, molds, actinomycetes, yeasts, etc. Humidity and temperature of storage as well as original cleanliness of the grain, of the mills, and storage places affect the microbial content. Many molds occur in flour and can cause spoilage in the stored product; especially under humid conditions. During baking most of the vegetative forms of molds, yeasts and non-sporeforming bacteria are killed but bacterial spores and conidia of

\* This is grain, usually barley, which has been moistened and allowed to germinate. It is then usually dried. Germination develops the amylolytic (*amylon* is Greek for starch) enzyme, *diastase*. This hydrolyzes starches in the grains to simple sugars so that they can be fermented. Yeast cannot attack starch as such. Malt is also used, for the same reason, in making beer and liquors.

† Wort is an infusion of malt in water. It is largely a starchy fluid with other nutrients of the grain, plus its diastase—a rich, nutrient fluid for microorganisms of all sorts.

molds may survive. Temperatures inside the loaf of baking bread rarely rise much above 100° C.

**ROPY BREAD.** If bread is made with flour or other ingredients containing large numbers of spores of *Bacillus mesentericus* and related species, these will survive baking and may grow in the bread, producing a mucinous slime. When the bread is broken apart this slime is drawn into long threads resulting in a defective product called "ropy bread."

**MOLDY BREAD** is usually the result of extraneous contamination of cut surfaces or crust by hands, dust and knives, followed by holding under humid conditions at household temperatures. Slight surface growth of mold does not necessarily destroy the bread but gives it a musty odor or taste.

Since bread is not sterile it should be cooled promptly after baking. The wise housewife keeps bread in the refrigerator in warm weather. Wrapping bread in waxed paper helps keep the bread clean and prevents drying out but conserves a humid atmosphere and favors growth of molds and bacteria, unless refrigerated. Non-sporeforming pathogenic bacteria cannot survive proper baking of bread but may be introduced after baking by unsanitary handling.

### SOME FERMENTED FOODS

**Ensilage.** Fermentation of the carbohydrates in green plant tissues, especially by lactic streptococci, lactobacilli and *Leuconostoc*, is used constantly (and usually unknowingly) by farmers in the preparation of silage, a food for cattle. Finely chopped, partly mature plants like corn stalks or alfalfa are tightly packed in tall, cylindrical tanks (silos). Microorganisms of many kinds start to grow. The carbohydrates in the plant juices are soon fermented. As fermentation proceeds, the material becomes warm and acid. The heat can be reduced if the rate of oxidation is decreased (exclusion of free oxygen) by tight packing. Oxygen is used up rapidly so that molds and strict aerobes soon cease growth. Only facultative and strict anaerobes continue. The increase in acidity prevents the growth of putrefactive organisms. In the first stages probably the Enterobacteriaceae and other non-aciduric, but fermentative, microorganisms predominate. These are undesirable since they produce gas and often unpleasant flavors. As acidity increases they subside and the more aciduric lactic streptococci and *Leuconostoc* predominate. These produce more lactic acid, with small amounts of other products of fermentation which give an aroma and flavor to ensilage which is relished by cattle. The last stages of the fermentation and final increase in acidity are due to the very acidophilic and aciduric lactobacilli.

After three or four weeks, the process slows and the fermented mass gradually cools. Carbon dioxide is produced during the fermentation process and often settles in the lower part of silos so that a person ignorant of this may die if he stays in the depths of a poorly ventilated silo.

It has been suggested that fermentation in silos may be facilitated by introducing cultures of various fermentative bacteria such as *Streptococcus lactis* or *Lactobacillus* sp., as the material is packed. Under natural conditions various other organisms are doubtless involved, including the bacteria of the soil. In some sections of the country molasses is added to promote fermentation by the acid formers, and to improve palatability.



If too much soil is introduced with the fodder undesirable and excessive putrefactive processes spoil the product. For example, butyric-acid organisms like *Clostridium butyricum* get in and ruin the silage by producing butyric acid which makes it "rancid." The action of such organisms constitutes a "disease" of silage. *Cl. botulinum*, a soil anaerobe forming a deadly exotoxin, has also caused much damage to livestock by its growth in silos.

**Sauerkraut.** The production of this savory delicacy is dependent upon the lactic acid fermentation of cabbage by the bacteria normally present on the plants. Pure cultures of lactobacilli are sometimes used to aid the process. Commonly, however, the fermentation is allowed to proceed naturally. Salt is placed between the layers of shredded cabbage as it is packed. This inhibits undesired bacteria and draws out the juices of the cabbage. Except for the salt, sauerkraut is quite analogous to silage.

**BACTERIOLOGY OF SAUERKRAUT.** Only facultative, anaerobic, aciduric and acidophilic and thermoduric forms, not sensitive to 2 per cent salinity, can survive. During the first two to five days, species of *Leuconostoc*, especially *L. mesenteroides*, are common. Later, these die off, because of the acidity, and organisms of the genus *Lactobacillus*, because of their greater resistance to acid, gain the ascendancy. Species such as *L. plantarum* and *L. brevis* (pentoaceticus) are commonest, in the order named. The latter is most aciduric and predominates finally. Acetic and lactic acids, carbon dioxide and alcohol are produced in considerable amounts, by the heterofermentative *L. brevis* and probably by other heterofermentative microorganisms. Other acids and esters and diacetyl give pleasant aromas and flavors. Temperatures around 70° F favor the best fermentations. Higher temperatures may induce darkening due to abnormal fermentation.

**Pickles.** Fermentation by mixtures of organisms normally present, in a manner analogous to the manufacture of ensilage and sauerkraut, is an important part of the production of pickles, ripe olives and the like. In making pickles commercially, selected and sorted cucumbers are placed in large wooden vats and covered with brine solution of from 8 to 10 per cent concentration. A framework of wood is placed over the pickles to prevent their floating. As water is drawn from the vegetable by the brine more salt is added gradually over a period of about six weeks till the concentration is increased to about 18 per cent. In making dill pickles salt concentrations of around 5 per cent are used.

During the summer, i.e., when cucumbers are ripe, temperatures range around 28° C. Under these conditions, and at the salinities provided, active growth of halophilic, acidophilic and thermophilic organisms occurs. In pickle vats the most important of these are coliforms, lactics and yeasts. Most other organisms are inhibited by the brine and acidity. The succession of microorganisms is analogous to that seen in sauerkraut manufacture and involves many of the same species.

The coliforms, active for a few hours, are soon replaced by the lactics (especially *Lactobacillus plantarum* and *Leuconostoc* species). The yeasts are temporarily suppressed by the initial acidity. After 1 to 2 weeks the acid formers decline in numbers from over a billion per ml to only a few hundred thousands. Yeasts, especially *Torulopsis holmii*, *Torulaspora rosei* and *Hansenula subpelliculose*, while rarely outnumbering the lactics, greatly increase

in numbers as the acid formers decline. After 3 to 4 weeks numbers of all organisms diminish.

The action of the lactic acid, brine and combined microbial growth and metabolism, changes the color, consistency and flavor of the cucumbers. After 8 to 10 weeks the vat is emptied and the pickles, now called "salt stock," are packed in fluid containing vinegar, sugar and various flavorings, spices, dill, etc.

It is evident that halophilic and aciduric organisms capable of growing at temperatures of 25° to 30° C should predominate. If temperatures are low, around 20° C, and the salinity only about 5 per cent, undesired slimy growths may occur. Slime-forming organisms such as *Leuconostoc* and sporeforming bacilli, and pectinase formers which destroy vegetable tissues, and other hydrolytic species, may then ruin the pickles. Stock to be used for dill pickles (low in acid and salt) is particularly liable to such spoilage.

### PRESERVATION OF FOODS

Methods may be divided into two general classes: (1) bactericidal methods, resulting in the immediate sterilization (or "virtual sterilization" or "commercial sterilization") of food; (2) bacteriostatic methods, which restrain the growth of microorganisms for long periods but which do not, under the usual conditions, sterilize the food. From a practical standpoint, canning or such processes as making jams, jellies, and preserves, chemical preservation, and smoking, are the principal methods of the first type. The use of microbicidal radiations will probably take its place among these time-honored methods in the future. Bacteriostatic methods include: drying, freezing, refrigeration at temperatures slightly above freezing, various types of pickling and more recently, the use of antibiotics.

**Canned Foods.** Modern home or commercial canners have to consider not only the killing of bacteria likely to cause spoilage or disease but also the effect of the processing on the palatability and appearance of the food. If canned foods are processed long enough they can be absolutely sterilized. This may require so many hours of heating in steam under pressure (especially non-acid foods) that the palatability and qualities of the foods suffer: they become mushy and discolored and have bitter flavors. Prolonged heating also adds to the cost of canning. The aim of the canner, then, is to heat as little as possible, consistent with safety from food poisoning and spoilage.

From the standpoint of food poisoning the only organism likely to resist autoclaving is *Clostridium botulinum*. Even the most resistant spores of this species are killed by autoclaving at 121° C in fifteen minutes, at pH 7.0.

A good margin of safety in time and temperature of processing must be left for the possibility of some super-resistant or protected spores. Actually, in order for the center of solid masses, like meat and sweet potatoes in a can, to reach 115° C, the whole can must be held at that temperature or higher for a considerable time—possibly an hour or more, depending on the size of the masses and of the can and the amount of circulation of fluid in the can, as well as insulating air pockets. In commerce, air pockets are largely eliminated by evacuation before sealing the cans. Also, the food is already hot when placed in the cans.\*

\* Actual times and pressures to be used in home canning can be obtained from various reliable Bulletins on Home Canning, available from the Superintendent of Documents, Government Printing Office, Washington 25, D.C.

Acidity greatly reduces the time and temperature necessary to preserve foods by heat even though they may contain resistant spores. Canned tomatoes, rhubarb, and acid fruits (pH 3.5 to 4.5), for example, require only a few minutes at 100° C to preserve them. Nearly neutral materials like meats, corn, spinach, and beans require much longer periods (up to 1 hour) at autoclave temperatures (121° C). *Cl. botulinum* ordinarily will not grow if foods have an acidity greater than pH 4.5. Some foods, which might be ruined by long processing, are therefore acidified to a greater degree than pH 4.5 and processed for a much shorter period. The acidity not only reduces danger from spoilage and botulism in the stored food but makes the short heating more effective.\*

Spoilage of canned foods is not always dangerous. Food may sour or putrefy yet cause no disease. On the other hand, if one spoilage organism is present and can grow, another may be present also, and this one may be the dangerous *Cl. botulinum*, cause of botulism.

**BOTULISM.** In meat sausage, especially the softer, moist sausages, as well as inside of cans of meat, vegetables and fish, strict and facultative anaerobes may find good pabulum and good anaerobiosis. The bacteria get into the containers when foods having soil or manure on them, visible or not, or when unclean containers, are used. If the cans are not sufficiently processed and if storage (as is usual with canned goods) is at household temperatures, these anaerobic bacteria can grow. They may be harmless. On the other hand, the deadly *Cl. botulinum* may be present. Sometimes the growth is not sufficient to "spoil" the food perceptibly and it may be eaten. *Clostridium botulinum* gives off a very potent exotoxin. The toxin of *Cl. botulinum* is poisonous when swallowed by man or animals. It is apparently unaffected by the acidity or pepsin of the gastric juice as are most other bacterial toxins. It is said to be the most deadly biological poison known. Botulinal toxin is absorbed directly from the stomach and intestines. It affects the nerve-muscle complex, producing a flaccid paralysis, particularly of the face, eyes and throat and respiratory system. As in diphtheria and tetanus, after advanced symptoms appear antitoxin is of greatly lessened avail therapeutically.† Botulism has a high death rate, around 68 per cent. The four home-canned foods most commonly responsible for botulism are corn, beans, beets, and asparagus. Note that none is an acid food and that three of them are often contaminated with

\* New methods of heating large masses throughout in a few seconds by radiation with penetrating infra-red heat waves may revolutionize food processing. The passage of high-voltage electrons is also said to sterilize food. Both of these processes are still in an experimental state of development. They promise to develop into million-dollar industries.

† There are five serological types of botulinal toxin: A, B, C, D and E. Type A is the most common in the United States. There is a specific antitoxin for each type, but polyvalent antitoxin is commonly used in treatment.

A simple test for botulinal toxin in foods, blood or stomach contents of victims is to inject the suspect material into 3 mice intraperitoneally as follows:

*Mouse a*—1 to 2 ml of suspect material heated at 90° C for ten minutes. This mouse should survive, since botulinal toxin is destroyed at 90° C in ten minutes.

*Mouse b*—1 to 2 ml of suspect material, unheated. This mouse should show typical symptoms of botulism if the toxin is present.

*Mouse c*—1 ml of polyvalent antibotulinal antitoxin. One hour later, 1 to 2 ml of the same material as mouse b. This mouse should survive with no symptoms.

soil. Commercial foods in the United States are exceedingly rare as the cause of botulism.

**PRECAUTIONS IN HOME CANNING.** In home canning, much can be done toward obtaining safer, more palatable and better looking foods if a few simple precautions are taken:

1. Use only sound foods, fresh as possible, well washed and clean. They should be boiled in a covered vessel, with just enough fluid (water or juice) to cover them. This should continue for at least fifteen minutes, or until they are heated throughout. This will kill many spores.

2. With a clean ladle, transfer the hot food into clean (boiled) preserving vessels and cover as directed by the manufacturer of the container. Do not pack too tightly. Do not fill more than four fifths full. *Fluid must be able to circulate freely.*

3. Place the vessels promptly, while hot, in the sterilizer. If fractional sterilization (tyndallization) is to be used, any covered vessel, such as a wash boiler, may be used. The water in the sterilizer should be boiling. The food should be processed in the boiling water or steam for 1 hour on three successive days. Covers should be loose to relieve pressure.

A pressure cooker is quicker and more effective.

4. Manufacturers' directions generally accompany pressure cookers. If you have no directions, then, for general purposes, proceed as follows:

- (a) *Be sure the safety valve, air vent, and pressure gauge are in working order or you may have a bad explosion.*

- (b) Open the air vent wide. Have about one inch of water in the bottom of the cooker.

- (c) Clamp on the cover tightly in the correct position (see markers on cooker and lid).

- (d) Leave the air vent open until *pure steam* issues with a *loud hissing noise*. Allow four minutes after this.

- (e) Close the air vent and watch the pressure gauge till from 12 to 15 pounds pressure is reached.

- (f) Adjust the heat source so that the pressure neither rises nor falls during at least thirty minutes (for quart jars and smaller sizes). Tomatoes, rhubarb, sour cherries and the like may be processed only 10 minutes.

- (g) At the end of the time of sterilizing, turn off the heat and *allow the pressure to subside completely*. Do not open the air vent until the pressure is down.

- (h) Open the air vent.

- (i) Remove the lid and the food vessels and tighten any loose covers.

Contamination with soil is especially to be avoided as it introduces thermophilic bacteria likely to cause spoilage in spite of heating, as well as *Cl. botulinum*. *Cl. thermosaccharolyticum* (No. 3814), an anaerobe, can cause gassy souring even of moderately acid processed foods if they are stored at warm temperatures at which the thermophil can grow. *Cl. stearothermophilum* (No. 1518) will resist any ordinary processing, being able to withstand 115° C for over two hours at pH 7.0. However, being a strict thermophil, it does not grow if the processed cans are promptly cooled and stored in a cool place.

Canned foods may frequently contain spores of such organisms, which are

viable but fail to grow under ordinary conditions of storage. These foods may be said to be "virtually sterile" or "preserved by heating" or "commercially sterile."

**Freezing.** The frozen food industry is now a very important one. While some microorganisms in foods are killed by freezing, considerable numbers survive and a few grow at or below freezing temperatures, but do so very slowly. Food-borne pathogens, if present, may survive freezing for weeks if the foods are not too acid (less acid than about pH 6.0). Most foods remain practically unchanged for months while frozen at about  $-26^{\circ}\text{C}$ .<sup>\*</sup> Autolysis and microbial decomposition are reduced to virtually nil. On thawing, foods may undergo rapid deterioration because the tissues are dead or inactive and the natural resistance of fully active tissues is reduced or absent. Microbial decomposition can set in with little delay at room temperature.

As in canning, only whole, sound, clean, fresh foods should be selected. Freezing must be prompt and rapid to be effective in stopping autolysis and microbial growth, as well as in preventing loss of flavor and vitamin content. Many foods may be "blanched" (scalded or immersed in boiling water for a few minutes) to destroy autolytic enzymes. This also kills some organisms on the outer surfaces.

Most frozen foods are virtually the same as fresh foods so long as they remain frozen but when thawed are more perishable. Once thawed, they should be used immediately.

If stale or heavily contaminated foods are used for freezing, spoilage will obviously be much more rapid both in the frozen state and after thawing. Further, the danger from food infection and/or food poisoning is greatly increased by the use of such foods. This is because pathogens, if any were present to begin with, have had an opportunity to grow prior to freezing and are greatly increased in dosage (Chap. 24), both of live cells and of exotoxins. These are nicely preserved by the freezing process! The same considerations apply as well to frozen dairy products as to frozen meats, vegetables and other foods.

Interruptions to electric current supplying frozen-food lockers may cause serious damage if prolonged for forty-eight hours or more in hot weather, since microorganisms, including dangerous pathogens like *Cl. botulinum*, may grow during the time the food is not frozen. Their activities may not be readily apparent when the refrozen food is later thawed by the consumer. Indeed, food refrozen after being thawed unknown to the consumer, may be a very dangerous product. The complete preliminary *cooking of foods to be frozen* goes far to eliminate all microorganisms and prevents rapid spoilage after thawing. The use of clean paper packages is also a good sanitary precaution. A sure means of making any food safe from infectious organisms and from botulinal toxin is to heat all of it to  $100^{\circ}\text{C}$  for at least fifteen minutes just before eating.

Food poisoning (botulinal or staphylococcal) and/or food infection (salmonellosis, shigellosis, etc.), due to commercially frozen foods properly processed, are virtually unknown in the United States. A few rare occurrences appear to have been due to accident or misunderstanding.

<sup>\*</sup> This is approximately the temperature of the home freezing locker.

**Refrigeration.** Most microorganisms of food spoilage grow very slowly at around 5° C, the temperature of the average household electric refrigerator. However, few are killed by such temperatures. Refrigeration is an excellent example of bacteriostasis. Foods of the most perishable sort remain edible and unspoiled for days or even weeks at 5–6° C, but eventually develop sour, offensive tastes and odors and undergo drying, loss of flavor and slow decomposition. (See also section on Influence of Refrigeration Temperatures earlier in this chapter.)

Every refrigerator should have a thermometer in it, since an inefficient machine not only wastes food and ice or electricity but may give a false sense of security.

**Chemical Preservatives.** Many so-called chemical preservatives were formerly used to check microbial growth in foods. Formaldehyde, salicylates, boric acid, hypochlorites and most others have been prohibited by law as undesirable or harmful. The two most commonly in use today are sodium benzoate and sulfur dioxide or sodium sulfite. Some states prohibit their use. Probably neither is harmful in the concentrations used (0.1 per cent benzoate and up to 3,000 ppm sodium sulfite). They act mainly to prevent growth and do not necessarily sterilize food. Benzoate is used in catsup and some other vegetable products, such as cider, etc., which are not heat processed, or which are treated by milder heating processes resembling pasteurization. Benzoate is especially effective in acid foods. Sorbic acid has been used to advantage as a substitute in low-acid pickles though it, too, is more effective in more acid solutions. Sulfite exerts some antimicrobial action and also preserves color of dried fruits.

Sodium nitrate or nitrite, or mixtures of these, are usually added to sodium chloride in mixtures for curing meats (corned beef, ham, etc.). Whether the nitrates or nitrites have any bactericidal or bacteriostatic action per se seems to be undecided, but meat packers find less spoilage when these salts are used. They appear to have an adjuvant action in acid solutions (most cured meats are acid, pH around 5.8) and on the preservative effect of NaCl. Nitrites and nitrates are particularly desired because they give good red color to meat.

**ANTIBIOTICS.** The use of antibiotics has been discussed previously in this chapter.

**Smoking.** This is a time-honored means of preserving meats and fish. Foods to be smoked are usually salt-cured first, to prevent rapid deterioration since smoke curing is slow. The preservative principles in wood smoke are various cresols, a mixture of formic, acetic and other organic acids and alcohol ("pyroligneous acid"), and formaldehyde; all of which are bactericidal substances. These are gradually absorbed in small quantities by the tissues exposed to the smoke. The meat is thus "cured" and rendered impervious to the action of most microorganisms, as well as given an agreeable aromatic flavor. Kept dry, it is preserved almost indefinitely. In warm, humid weather it may become moldy and rancid, especially on the surface.

**Drying.** This method of preserving food is perhaps as old as the human race. It is well exemplified by making hay, raisins, "jerked" meat, etc. Exposure to the sun adds the bactericidal effect of ultraviolet to such procedures. Drying per se is largely, but not entirely, a bacteriostatic method. Dried foods, unless preserved with such materials as salt, sugar, sulfite, etc., are not stable



in humid conditions. The flora is much the same as that of fresh foods. The production of dried egg powders, dried milk, dried salted fish, dried sugared fruits, etc., has developed into large industries.

Drying of many meats, fish and fruits is often done in bright sunshine. However, to save time, and prevent contamination and loss of color and vitamins many foods are dried in warm ovens, or, if fluid, like milk, by spraying through warm, dry air onto warm, revolving drums. Overheating is a great problem in such processes. So are thermophilic microorganisms.

**Preservatives Commonly Classed as Foods.** **SALT.** For hams, fish, etc., to be smoked, salt is applied either in the form of strong (20–25%) brine in which the foods are soaked for some days, or in the form of a dry mixture (also containing sugar and flavor) which is rubbed into the ham or fish: salted fish, “sugar-cured” hams. The sugar and salt diffuse through the meat, withdraw much fluid and cause the meats to shrink and become drier and firmer. The color is fixed. Growth of microorganisms is inhibited by the salt and sugar concentration. Often the salt-sugar or brine solution is injected with trochars into the tissues, especially along the bone of hams. “Bone-taint” is prone to occur due to bacterial action at the center of the meat, farthest from the surface action of chilling, curing or smoking. Pickling solutions may also be injected through the vascular system of the animal after slaughter, before it is cut up.

If large numbers of halophilic or other bacteria are introduced by contaminated pickle solution or instruments, the entire mass may spoil, sour, become gassy, rancid or putrid, depending on the kinds and numbers of organisms introduced. Dirty salt will, of course, introduce heavy contamination to any food receiving it. Cleanliness in food packing plants pays big dividends in money saved.

Halophilic organisms often grow in salt brines used for preserving foods. This includes yeasts, mold and bacteria. Some of these cause desirable flavors or they may cause putrefaction.

Some workers believe these halophilic organisms to be merely variants derived from ordinary saprophytic species of soil, water, dust, etc. by a process of adaptation to higher osmotic pressures. There is considerable evidence to support this view. In many industrial processes, as in food processing, there appear to be microorganisms peculiarly adapted to grow in the conditions of any given process, and many have names and identities which are familiar mainly to workers in that industry. Examples are the organisms inhabiting meat pickle vats, the thermophiles in fermentation vats in fermentation industries, the cryophilic organisms of refrigerator plants, and so on.

**SUGAR.** To be effective, the osmotic pressure of solutions of sugar or salt must be greater than that of the microorganisms. Sugar solutions of 50 to 70 per cent concentration effectively prevent most growth. A very few organisms (some yeasts and molds) may grow slightly in such material. In humid atmospheres foods preserved by the higher concentrations of sugar absorb enough water superficially from the air to dilute the sugar and permit surface growth of some molds and bacteria.

Many microorganisms may survive for long periods in such solutions and in honey, molasses, etc., and may even grow slowly to some extent. In preserving fruits, etc. with syrup it is advisable to heat the syrups and scald the fruits, or heat both together thoroughly, to kill the bulk of the microorganisms

initially present. Exclusion of air, as by paraffine or tight covers on jars of preserves, will prevent growth of adventitious aerobic organisms, especially molds, on the surfaces.

**SPICES.** Spices used for flavor are often heavily contaminated with soil and dust flora. Most spices have no inherent bactericidal or bacteriostatic activity, in spite of a centuries-old tradition to that effect. Some food packers treat contaminated spices with gaseous disinfectants (carboxide, ethylene oxide) before using them.

**ACIDS.** Acids occurring in foods as a result of natural fermentation or souring are, principally, lactic as in cheese, buttermilk, etc.; and acetic as in vinegar. Usually there are present also in these products small amounts of various alcohols, glycerine and some other acids. Acids in ripe tomatoes and fruits include citric, malic, tartaric and the like. All have a preserving action except against certain aciduric molds and organisms such as lactobacilli.

**Foods in the Kitchen.** Aside from considerations of cleanliness, sanitation and the proper processing of preserved foods, which have as their main objective the prevention of disease, the domestic economist is interested in saving from spoilage foods bought in quantities at lower-than-usual prices, or bought only during weekly or monthly trips to food shops. She is also interested in "left-overs." The surest mark of high achievement in the art and economics of the kitchen is the skillful and appetizing use of foods which are kept from one meal to another—the pot of good soup (excellent bacteriological medium!) which is saved for tomorrow's dinner; the left-over porridge or bread pudding, and so on. All are valuable assets to the resourceful domestic economist. But they are also subject to spoilage by microorganisms from dust and utensils unless those organisms are killed or kept from multiplying.

The household refrigerators or freezing cabinets offer the readiest means for saving foods. When these places are overcrowded, or are for other reasons not available, spoilage can be retarded by reheating foods in covered vessels and not opening the vessels until more of the food is needed (Tyndallization). For example, the pot of soup, stew, vegetables or other non-acid foods may be brought to a boil for a few minutes in a vessel with a good, dust-tight cover on it. The heat kills any vegetative forms of microorganisms and the spores of "wild" yeasts and molds that might have gotten in while it was cool and uncovered. A good many bacterial spores will be killed also, but some will survive. However, these probably will not grow sufficiently to cause spoilage for eighteen hours or more. If the soup should be brought to a boil the next day, the cover not having been removed in the meanwhile, then virtual fractional sterilization has been accomplished and the food should keep for some time. The importance of re-cooking as a means of preventing certain kinds of food poisoning has previously been emphasized.

With solid foods not immersed in water, such as a roast of meat, spoilage tends to occur mainly on the surfaces. Much of this is introduced by knives, forks and other utensils. Clean utensils introduce less contamination than soiled ones. If the cooled roast is re-heated in a very hot oven for a few minutes after the meal, so as to cook only the exterior, and is left in the oven without opening the door, thus avoiding re-contamination from the air, surface growth is definitely checked and the meat may remain good for some days, depending on the extent and depth to which initial contamination was

introduced, and the temperature of the kitchen. In reheating meat, bowls of pudding, etc., in this manner it is desirable to use a "self-basting" roaster or other covered dish to prevent drying out of the food. The surface reheating should be for a short period, say ten to fifteen minutes, at high temperature.

If food is not reheated it should be promptly placed in an efficient refrigerator. It is poor practice to allow food to stand in the warm kitchen overnight, incubating microorganisms into the millions, and then put it in the refrigerator next morning. This is locking the barn after the horse is stolen. Put all left-overs in the refrigerator *immediately* after a meal.

### BACTERIOLOGICAL EXAMINATION OF FOODS

**Quantitative Microbiology of Food.** Estimating the total numbers of bacteria in milk, water, ground meat and the like, involves basically the same procedures regardless of the product. The main differences in procedures lie in methods of collecting, preparing and measuring the sample. These steps are guided by the nature of the material. In every case efforts are directed toward washing, scraping, shaking, comminuting in a "Waring blender," or in some other manner getting the microorganisms of the sample of food *into a fluid suspension*. This can then be diluted and put into Petri plates with clear agar for colony counting. A water-clear inoculum is needed for plate counts since particles confuse colony enumeration. For inoculation into tubes of broth clarity of inoculum is not so essential.

In general it may be said that the plate-count method for examining milk is the prototype of *quantitative* methods for examining food products in general. However, the direct microscopic examination of the sample is also very useful, both quantitatively and qualitatively.

**Qualitative Microbiology of Food.** The examination of water for the coliform group is typical of *qualitative* procedures in examining foods for this group.

The nature of the medium used, the temperature (thermophilic, mesophilic or psychophilic) and conditions of incubation (aerobic or anaerobic) and other factors (acidity, salinity, etc.), must be modified to suit the flora. The errors in food microbiology are in most respects similar to those inherent in the enumeration and identification of organisms in milk and water. The plate-dilution count may be made both quantitative and qualitative by using a medium, incubation temperature or other factor selective for only one species or group of species. For example, acid-formers may be detected by adding glucose and an indicator to the nutrient medium used for plating. The numbers of bacterial spores present may be estimated by heating the material to 80° C for twenty minutes before plating. Other selective procedures will occur to the ingenious microbiologist and can be adapted to various problems.

Fecal streptococci appear to be a more dependable indicator of fecal contamination in several frozen foods, notably fish and citrus products, than are coliform organisms. The coliform organisms are so ubiquitous that their presence in many foods does not accurately indicate fecal pollution at all. Further, they do not survive in acid foods as do the enterococci. Enterococci are easily isolated and identified by using selective media containing such agents as sodium azide and crystal violet to inhibit undesired organisms.

**Opening Containers.** In examining foods packed in containers, as canned

goods, oysters in shells, etc., it is obvious that if the outer surface of the container is not thoroughly cleaned and sterilized before piercing, organisms will be introduced from the exterior, at the moment of opening, into what might otherwise be a sterile product. If there is a removable cover on the container every effort should be made to remove dirt and dust, particularly around the edges close to the mouth of the container, as it is here that contamination is most likely to be introduced. Painting with sterile paraffin or shellac may immobilize dirt or dust on the outside of the container so that it cannot enter. Preliminary thorough scrubbing with a brush, application of disinfectants and washing with a fast, fine stream of hot sterile water are useful. The wash water should be *completely* removed and the surfaces dried before opening the vessel.

The contents of cans can best be removed (with syringes if fluid; sterile forceps if solid) through a small opening, the site for which is sterilized by the application, for five to seven seconds, of an intense, needle-blast flame, or by thorough chemical disinfection. Sterile openers should be used. In heating cans, internal pressure may be generated, especially if the can contains gas from spoilage. As there is danger from explosion, care should be exercised in such work.

#### FOOD SPOILAGE

The occurrence and type of spoilage of food will in large part depend on (a) moisture; (b) temperature of storage; (c) presence and species of viable organisms; (d) pH of the food; (e) chemical composition of the food; (f) nature of preservative agent, if any.

**Specific Causes of Spoilage or "Diseases" of Foods.** Sometimes conditions in food manufacturing or preserving processes become especially favorable to the luxuriant growth of a single species of organism. This can then dominate the flora of a given food, or of a preserving or pickling fluid, and may produce some striking alteration of taste, odor, pigment, sliminess, or the like. A good example is "ropy bread" due to the use of flour heavily contaminated with spores of *B. mesentericus*. Canned oysters sometimes become contaminated with a red torula in a dirty shucking house in which growth of this red species has gotten well distributed. The oysters and fluid in the cans are covered with the red growth and not salable although no marked change in odor or taste is produced and the red torula is presumably harmless when eaten. Similar organisms sometimes grow in sauerkraut. Butter sometimes acquires a rancid, fishy or oily taste or odor because of the growth in it of large numbers of some particular organism which hydrolyses fat or produces these bad-smelling and bad-tasting compounds. Perhaps a dirty churn may have heavily seeded the whole lot of butter. Sometimes a red, blue or yellow or other color is imparted to milk, fish, etc., by heavy growth in it of a *Serratia* species, a *Pseudomonas* or a *Flavobacterium*. Various spottings, ammoniacal decompositions and peculiar types of fermentation are produced under special circumstances and by specific organisms. These spoilages are usually due to the use of a stale product to begin with, or to a dirty tank, implement or preservative heavily contaminated with some organisms which can grow in the particular food or circumstances involved.

Usually the control of such specific types of spoilage is not too difficult, once the organism is isolated and its growth peculiarities and source studied. However, spoilage often presents very abstruse and challenging problems for the graduate student.

Contamination with mixtures of organisms found in soil, dust, decks of fishing boats, floors of slaughter houses, canneries, dairies, etc. is really the most difficult to control. It cannot be dealt with by means of a single, specific measure designed especially to eliminate one particular source of difficulty such as a single, contaminated piece of machinery. It must be eradicated by measures of general cleanliness. These may involve expensive manpower, steam and hot water, general disinfection on a very wide scale, and costly measures against continuous reintroduction of dirt.

#### REFERENCES

- Allen, C. H., and Fabian, F. W.: Comparison of *Esch. coli* and *Strep. faecalis* as a test organism to determine the sanitary quality of food. *J. Milk and Food Tech.*, 1954, 17:234, 237.
- Bausch, E. R., and Goresline, H. E.: An Analysis of Commercial Frozen Egg Products. U. S. Dep't Agri. Circ. No. 932, 1953.
- Bellamy, W. D., Goldblith, S. A., Colovos, G. C., and Niven, C. F., Jr.: Symposium on applications of ionizing radiation to food and pharmaceutical preservation. *Bact. Rev.*, 1955, 19:266.
- Dack, G. M.: Food Poisoning. 3rd ed. Univ. of Chicago Press, Chicago, Ill., 1956.
- Dawson, E. H., Gilpin, G. L., and Reynolds, H.: Procedures for Home Freezing of Vegetables, Fruits, and Prepared Foods. Agricultural Handbook No. 2, U. S. Dep't of Agr., 1950.
- Etchells, J. L., and Jones, I. D.: Procedure for bacteriological examination of brined, salted and pickled vegetables and vegetable products. *Am. J. Pub. Health*, 1946, 36:1112.
- Expert Committee on Environmental Sanitation, 4th Report. World Health Org. Tech. Rep. Ser., 1956, No. 104. Columbia Univ. Press, New York.
- Gaughran, E. R. L.: The thermophilic microorganisms. *Bact. Rev.*, 1947, 11:189.
- Goresline, H. E.: A discussion of bacteriological standards for dehydrated foods. *Am. J. Pub. Health*, 1947, 37:1277.
- Greathouse, G. A., Wessel, C. J., and Shirk, H. G.: Microbial deterioration of manufactured materials. *Ann. Rev. Microbiol.*, 1951, 5:164.
- Gunderson, M. F., Kyle, T. S., and McFadden, H. W.: The Bacteriology of Commercial Poultry Processing. Burgess Publishing Co., Minneapolis, Minn., 1954.
- Hartsell, S. E.: The longevity and behavior of pathogenic bacteria in frozen foods: The influence of plating media. *Am. J. Pub. Health*, 1951, 41:1072.
- Jensen, L. B.: Microbiology of Meats. 3rd ed. Garrard Press, Champaign, Ill., 1954.
- Larkin, E. P., Litsky, W., and Fuller, J. E.: Incidence of fecal streptococci and coliform bacteria in frozen fish products. *Am. J. Pub. Health*, 1956, 46:464.
- Nickerson, J. T. R., Proctor, B. E., and Goldblith, S. A.: Public health aspects of electronic food sterilization. *Am. J. Pub. Health*, 1953, 43:554.
- Silverman, M.: New ways to keep food fresh. *Sat. Eve. Post*, 1956, 228:36.
- Spencer, R.: Hygiene and sanitation in the fish industry. *Roy. Soc. of Health J.*, 1957, 77:41.
- Standard Methods for the Examination of Dairy Products. 10th ed. Am. Pub. Health Assoc., New York, N. Y., 1953.
- Standard Methods for the Examination of Water, Sewage and Industrial Wastes. 10th ed. Am. Pub. Health Assoc., New York, N. Y., 1955.
- Stewart, J. A., and Clark, B. S. (Pilcher, R. W., Ed.): The Canned Food Reference Manual. Am. Can. Co., New York, N. Y., 1947.
- Symposium, First International, on the bacteriology of non-sterile meat packs. Editions Medicales Flammarion, Paris, 1955.

Various Authors: Eggs and Egg Products. Special Report No. 60, 1954. Food Investigation Organization, Dep't of Scientific and Industrial Research (London). H. M. Stationery Office, London.

Vaughan, R. H., and Murdock, D. I.: Sanitary significance of microorganisms in frozen citrus products. *Am. J. Pub. Health*, 1956, 46:886.

Wedberg, S. E.: *Microbes and You*. The Macmillan Co., New York, 1954.

Wolford, E. R.: Certain aspects of the microbiology of frozen concentrated orange juice. *Am. J. Pub. Health*, 1956, 46:708.

## Microbiology and Industry

MICROORGANISMS ARE being made use of more and more widely in medicine, agriculture and the industries and are more and more being regarded as the servants of man.

### KINDS OF INDUSTRIAL PROCESSES

Industrial processes based on microbial action may be grouped under six general headings:

1. Cultivation of *the organisms themselves* (principally yeasts, some algae) for use as food. This is not as yet a large industry in the United States where other foods are plentiful.

2. Cultivation of microorganisms so that the *enzymes* or other substances which they secrete (amylase, protease, antibiotic, etc.) may be collected, purified and sold for commercial or medical use. Some aspects of this type of process are fairly extensive.

3. Cultivation of microorganisms under conditions in which they decompose (ferment\*) various substrates (usually carbohydrates), *the products of the fermentation* (various alcohols, organic solvents, lactic acid, citric acid, etc.) being recovered, purified and sold. Rather extensive industries are based on such processes.

4. Cultivation of microorganisms in food products (mainly fermented vegetable products and dairy products) for the purpose of *producing certain flavors, consistencies, nutritive values*, etc., in the products. These are large industries.

5. Use of microorganisms for *special industrial purposes* such as removal of sulfur compounds from petroleum, vitamin assay, etc. These have limited, highly specialized applications.

6. The *uncontrolled action of mixtures of microorganisms* in processes like retting of flax, preparing hides for leather, coffee-bean hulling, etc.

\* The term fermentation, strictly speaking, applies only to anaerobic decomposition of carbohydrates. As used in industry it means any process in which microorganisms, aerobic or anaerobic, by their metabolic activities convert a raw material (carbohydrate, protein or other substrate) into a desired product. Thus, we hear of the lactic fermentation, the acetic fermentation (strictly aerobic), the butyric fermentation (anaerobic) and so on. In this chapter we shall use the term in the commercial sense.

**FACTORS OF IMPORTANCE IN DEVELOPING AN INDUSTRIAL PROCESS'**

In developing an industrial process based upon the action of microorganisms a great many details must be given consideration. Important among these are:

**1. Purity and Nature of Cultures.** It must be ascertained whether absolutely *pure* cultures *must* be used, or whether the mere *predominance* of one organism is sufficient. This may be a deciding factor, as the cost of preparing and maintaining pure cultures and sterile apparatus, etc., throughout a process may be excessive. For purposes of the present discussion let us assume a pure culture to be requisite.

The organism must be able to grow well in the medium to be used and under the conditions of the process. This will entail very exact studies of *optimum* conditions of aerobiosis or anaerobiosis, temperature, nutrition and pH. Valuable preliminary information on these points can be gained from experimental use of synthetic media of wholly known composition. Appropriate adjustments of the process and apparatus must be made to provide the necessary conditions.

The organism must be one which will evolve the desired end products or produce the desired result in the medium and under the conditions furnished, in amounts sufficient to yield a profit. Some firms have "pet" strains of microorganisms which excel in producing certain products, such as butyl alcohol, or itaconic acid, and which they have "developed" (selected variants) for these purposes. It has been found possible to induce industrially valuable mutations in microorganisms by ultraviolet radiations (see Chapter 15). Where sexual processes are known to occur the "breeding" of yeasts and molds for similar purposes is analogous to breeding of cattle for beef or milk.

**2. Medium or Raw Material.** A second factor to be considered is the substrate or medium. It should support luxuriant growth of the organism to be used and it must be available constantly at costs compatible with profit. Expensive handling machinery may be needed for some substrates.

An important item is the possible necessity of a preliminary treatment such as liming of very acid yeast "slops," distillery wastes, molasses and whey. Some substrates, such as sawdust or fiber, may need preliminary "digestion" with hot acid or alkali to hydrolyze them to fermentable substances. This all adds to the expense and time.

**3. Nature of the Process.** The more complicated and exacting the system of cultural details and preliminary heatings, dilutings and digestions, as well as the type of machinery ("cracking" stills, tanks, pumps) to handle the end- and by-products and the final wastes, the greater will be the cost and therefore the less the commercial practicability of any process. Any time-consuming "aging" or "ripening" processes eat into the credit side of the ledger. Sometimes very desirable end- or by-products may be found in commercial fermentations, yet the cost of their recovery may be prohibitive.

**4. Preliminary Experimentation.** The microbiologist working with 10-ml test-tube cultures may find many valuable things. When attempts are made to reproduce the test-tube experiments on a 100,000-gallon factory scale, however, the laboratory discoveries often fail to yield the promised result. Any process developed in the experimental laboratory must next prove its worth in the factory. A small-sized model or "pilot" plant is usually tried



after the preliminary laboratory work. All may depend on such a seemingly far-removed detail as international relations. These may affect the cost of importation of some raw product essential to the process under investigation. Then the industrialist turns to home resources, goes to Washington, or employs a resourceful microbiologist!

The whole matter is a complex of microbiology, chemistry, engineering and economics. Only the microbiology can be discussed here. Many chemical and microbiological processes in use at present are patented and secret, and specific strains of bacteria, yeasts and molds, which are zealously guarded, are often carefully developed in the laboratories of manufacturing concerns.

### SOME MANUFACTURED DAIRY PRODUCTS

Most commercial dairy products contain mixtures of microorganisms. However, pure-culture inocula (called "starter cultures") are essential to continued success in this highly competitive field. For these purposes *lyophilized*, desiccated, or liquid pure cultures of desired organisms may be purchased through dairy-supply houses.

**Maintenance of Starter Cultures.** In practice they are added, as nearly aseptically as possible, to about 600 ml (2/3 quart) of sterile or very-low-count milk (previously heated 30 minutes at about 88° C and cooled to 21° C) and incubated. This culture is called a "starter." It may be used to inoculate a batch of milk or cream for butter or cheese, or to inoculate a still larger lot of starter. Unless competent, wholly aseptic, bacteriological laboratory facilities are available in the dairy it is best to purchase new, pure culture for starter at least once a week; preferably oftener, otherwise the desired organisms are eventually displaced by worthless and harmful contaminants.

**Use of Starters.** For the best manufactured dairy products clean, fresh, high-quality (low-count) pasteurized milk or cream is essential. For use it is brought *quickly* to the desired incubating temperature. A large, *pre-emptive*, virtually pure starter inoculum of vigorously-growing, young cells of the desired lactic organism is added and thoroughly mixed with the milk or cream. Before the other bacteria in the milk have time to recover from their previous refrigeration or pasteurization and overcome their lag phase, the acidity quickly produced by the added lactic starter suppresses them. Further developments in the batch (butter, cheese, etc.) are dominated by the lactics and the dairyman.

**Interference with Starter.** (1) Milk from cows with infected udders (mastitis, garget) is very apt to contain enough antibiotic to inhibit the starter and ruin bacterial dairy products if the cows are being treated with antibiotics. (2) Another difficulty arises from bacteriophage lytic for *S. lactis*. These viruses are widely distributed and hard to control, though chlorine disinfectants will kill them. The best remedy is to obtain a 'phage-resistant strain of *S. lactis* to use in starter.

**Butter.** Most butter is made by churning cream which has been soured by means of cultures of *S. lactis*, *S. cremoris* and related organisms.

**SOURCES OF FLAVOR AND AROMA.** The pleasant flavor and aroma of butter is due mainly to *diacetyl*. This is not produced to any extent by *S. lactis* but by related species capable of attacking citric acid under acid conditions. *Leuconostoc citrovorum* and/or *Leuconostoc dextranicum* are com-

monly used for this purpose. They produce diacetyl from citric acid. Citric acid is normally present in small amounts in soured cream, or may be added to increase the flavor. Diacetyl has a pleasant "buttery" aroma. Smaller amounts of other bacterial products, such as acetic acid, also add pleasant aroma.

**Cheese.** Cheeses may be divided into three general types: (a) soft or cottage-type cheese and cream cheese (these are eaten in a fresh or unripened state); (b) hard- or rennet-curd cheese, including American, Roquefort, Cheddar, Edam, and Swiss or Emmenthal (these are "ripened" by the growth in them of bacteria or molds or both, which do not cause extensive proteolysis); and (c) soft or semisoft rennet-curd cheese, of which Camembert, Limburger and Liederkranz are types. These are ripened by more or less proteolytic organisms which soften the curd. The hardness of cheese depends

Table 30. *Types of Cheese.*

REPRESENTATIVE CHEESES	DISTINCTIVE ORGANISMS IN RIPENING FLORA
SOFT: Cottage Cream Limburger Camembert	} not ripened <i>Strep. liquefaciens</i> , etc. <i>Penicillium camemberti</i>
SEMI-SOFT: Blue (or bleu) Roquefort Gorgonzola	} <i>Penicillium</i> strains, like <i>P. roqueforti</i>
HARD Swiss Cheddar Parmesan	<i>Propionibacter</i> species Lactic group, <i>Geotrichum</i> Lactic group (brine cured)

to some extent on moisture and fat content as well as on heating and acidity of the curd, draining, salting, and conditions of storage. A list of common cheeses is given in Table 30.

**SOFT, ACID-CURD CHEESE.** In making cheeses of this type, represented by "cottage cheese," cultures containing mixtures of *Leuconostoc citrovorum*, *L. dextranicum*, *S. lactis*, etc., are added to pasteurized milk. These ferment the lactose, *Leuconostoc* adding flavor and aroma. The lactic acid which is thus formed coagulates the casein. Rennet may be added to hasten the coagulation and make the curd firmer. The curd is cut into small cubes. To firm the curd and separate it from the whey, the mass is heated slowly to about 50° C and held so for 30 minutes. Water is added; the curd settles. The water, with the whey, is drained off and the curd is pushed into heaps to drain. It is washed a second time with water and drained. About 0.5 per cent salt is added. Just before packaging many manufacturers add a little cream.

**HARD-CURD CHEESE.** In making hard- or rennet-curd cheeses such as Cheddar cheese, the milk is usually first acidified or allowed to become slightly

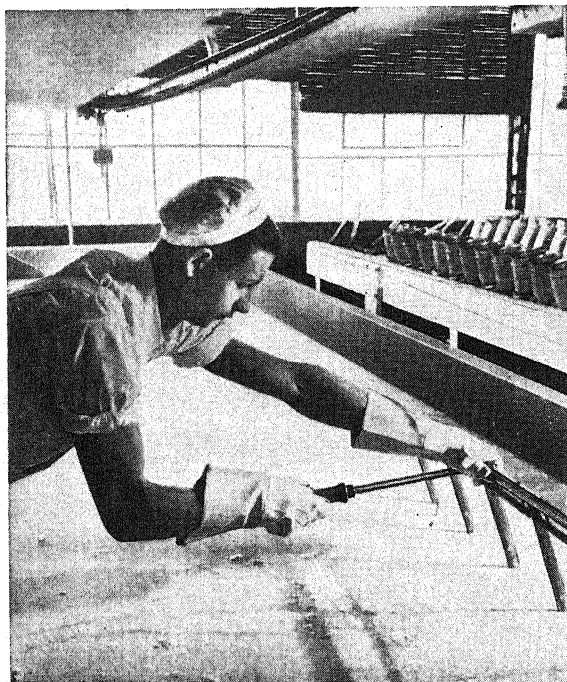


Fig. 44-1. Making Cheddar-type cheese. Liquid milk becomes "curd" and "whey" after "starter" and rennet are added. The milk forms into soft curd much like "Junket." This is firmed by heating gently. When it has reached the correct firmness, it is cut into small cubes ( $\frac{1}{4}$  inch) by special wire knives. The whey is slowly expelled from these cubes as the curd and whey are heated while slowly agitated by revolving paddles. The cubes of coagulated casein are then washed with water and drained. The picture shows the cutting operation. (Courtesy of Kraft-Phenix Cheese Corporation.)

sour. Cultures of *S. lactis* or *Leuconostoc dextranicum* are generally added for the purpose. Cheese "color" is added. After a slight acidity has developed rennet is added to make an elastic, rubbery curd which is later cut into pieces about one inch in diameter (Fig. 44-1) and heated. After heating to about 95° F the curd becomes firmer and the whey separates and is drained off and may be used for stock feed. In the preliminary stages, nearly all manufactured cheeses are much alike. Differences result from different methods of processing the curd: addition of different amounts of salt; special ripening; microorganisms; moisture; temperature and humidity of ripening, and other factors. The clumped masses of firm curd are chopped (*milled*) again, and piled up to press out whey (Fig. 44-2). This is called "cheddaring" in Cheddar\* cheese making. The curd is again milled, and then is salted, drained, and pressed in hoops to cure. Curing of Cheddar cheese goes on at about 15° C.

#### CURING OR RIPENING

**Hard Cheeses.** During the curing process of hard-curd cheeses of most types, whether Cheddar or others, various microorganisms, the varieties de-

\* Cheddar is the name of a town in England famous for this type of cheese making.

pending on the kind of cheese (e.g., *Str. lactis*, lactobacilli, *Geotrichum lactis*, *Aspergillus* and *Penicillium*), are allowed to continue a slow fermentative, lipolytic, and proteolytic action, the products of these processes yielding the substances responsible for the characteristic flavors, textures and aromas of various cheeses. Prominent among these are diacetyl, lactic, butyric, caproic, and acetic acids, and various amines, as well as various esters such as those which give flavors to ripe fruit juices. In addition, since many of these organisms synthesize vitamins, especially nicotinic acid, and vitamins of the B complex, the nutritive value of the cured (or ripened) cheeses is increased.

The *Escherichia*, *Aerobacter* and *Clostridium* and other rapid gas producers such as *Lactobacillus brevis*, are undesirable in cheese making since they produce gassy cheeses and "off" flavors; they may be especially active in the early stages. It has already been pointed out that *Propionibacterium* are active in the ripening and flavor of Swiss cheese. The "eyes" in Swiss cheese are due to the production of carbon dioxide by the bacteria, while its flavor is due in part to the formation of glycerol, propionic and succinic acids by these organisms. Propionates have the bitter-sweet taste distinctive of true Swiss cheese.

Much depends on the way the cheese is made. Swiss cheese is heated to between 45° and 50° C early in the process to prevent overgrowth of the streptococci and permit growth of the thermophilic lactobacilli (*L. thermophilus*, *L.*

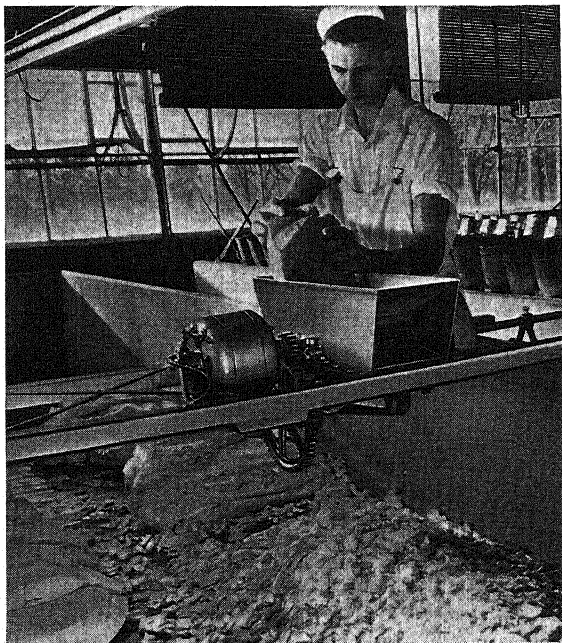


Fig. 44-2. Making Cheddar-type cheese. When cheddaring (firming and draining) are complete (judged by the firmness of the curd, its texture and acidity) the curd is milled (cut into small pieces) in preparation for salting and pressing. About 1½ pounds of salt are used to 100 pounds of curd. After salting, the curd is placed in cheesecloth or cellophane-lined "hoops" of the desired size and shape for pressing. (Courtesy of Kraft-Phenix Cheese Corporation.)

*lactis*, etc.) which give desired texture and flavor. These subside after a time and propionibacteria are then favored by incubation at lower temperature.

**Semi-soft cheese**, such as Roquefort or "blue", contains as high as 4 per cent salt and relatively little moisture. This prevents continued growth of most bacteria, as does also the low ripening temperature of 7 to 8° C. A species of mold (*Penicillium roqueforti*) is introduced by the inoculation of spores into the curd as it is put into the hoops for ripening. These grow in the interior producing the "sharp" flavor so characteristic of this type of cheese. As the mold is aerobic, perforations are made in the cheese to aerate the interior. The development of proper flavors and texture depends in great part on suitable conditions of temperature and humidity of the atmosphere for growth of the mold. In France, Roquefort cheese is usually made of ewe's milk which gives it a flavor and consistency different from Blue or Gorgonzola, which are made from cow's milk. The temperature and humidity (60%) of the atmosphere in certain caves in Roquefort is especially favorable for ripening. In the United States air-conditioning of some cheese ripening rooms is resorted to.

**The Curing of Soft Cheeses** like Limburger, Liederkranz and Camembert is due largely to the growth of organisms in a slimy coating on the outer surface. Numbers of microorganisms in this slime sometimes exceed 10 billion per gram. In Limburger, after subsidence of the initial acidity due to lactic organisms, yeasts begin to grow on the surface. They persist in the surface slime for about a week. *Bacterium linens* and *B. erythrogenes* (*Corynebacterium*-like species) then grow all over the surface, forming a reddish-brown coating commonly seen on many soft cheeses: Brick, Camembert, etc. *B. linens* and *B. erythrogenes* are important, proteolytic, widely-distributed dairy experts. The enzymes of these, as well as of other organisms (micrococci, yeasts, molds) in the slime, penetrate into the interior of the cheese producing the softening, and the *famous perfume*, of Limburger and similar cheese.

Camembert cheese is inoculated on the outer surface with a pure culture of the mold, *P. camemberti* (and/or *Geotrichum* (*Oospora*) *lactis*). These, with *B. linens* and other slime organisms, ripen the cheese from without inward. The outer portion is ripened first, has a firm, waxy consistency and forms a more or less thick, outer "crust" around the still "green," soft, inner portion.

#### VINEGAR AND ACETIC ACID; THE ACETOBACTER

Acetic acid is almost entirely responsible for the sour taste of vinegar. Indeed, a slightly sweetened, 3 to 5 per cent aqueous solution of acetic acid ("pyroligneous acid" or "wood vinegar") makes a marketable substitute for vinegar.

Commercial vinegar contains about 4 per cent acetic acid. The acid of naturally soured vinegar is derived from alcohol by the action of living organisms which are included in the family Pseudomonadaceae (genus *Acetobacter*). Pleasant flavors of natural vinegar are given by traces of various esters like ethyl acetate, and by alcohol, sugars, glycerin, volatile oils, etc., produced in small amounts by microbial action. Flavors are also derived from the fermented fruit juice, malt, or other alcoholic liquor (wine, beer, hard cider) from which the vinegar was made.

**In commercial vinegar-making** by biological methods, preliminary fermen-

tation of cider or wine to produce the necessary alcohol is often carried out by means of *Saccharomyces cerevisiae* (brewers' yeast). The *Acetobacter*, which act upon the alcohol (real, microbial alcoholics!), are strictly aerobic, non-sporeforming, pleomorphic organisms which, in barrels, form a thick scum or pellicle on the alcoholic liquid. They oxidize the alcohol to acetic acid as a source of energy, utilizing other substances in the fermented liquor as foods. In vinegar generators the alcoholic liquor trickles over the surface of inoculated, aerated shavings or other finely divided material (Fig. 44-3).

The *Acetobacter* are rods about  $0.5\ \mu$  by  $8.0\ \mu$ , although species vary in size. Branching involution forms and large, swollen cells frequently occur, especially in "mother-of-vinegar." Some species are motile. A species of historical interest is *Acetobacter* (*Mycoderma*) *aceti*, originally used by Pasteur to demonstrate the biological nature of vinegar formation. In practice, several species of *Acetobacter* usually act jointly. The over-all reactions probably are as follows:

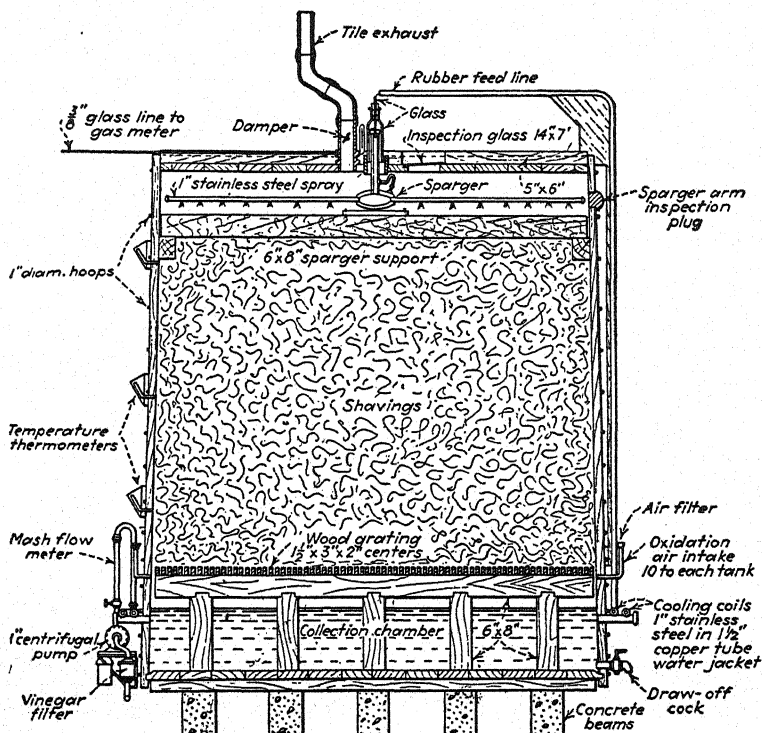
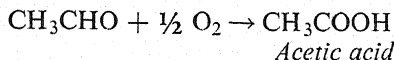
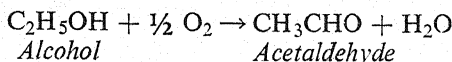


Fig. 44-3. Cross section of the Frings generator. The alcoholic liquor is sprayed over the shavings by the rotating stainless steel spray (sparger) near the top. Note the thermometers, cooling coils and air intakes. (Courtesy A. E. Hansen, Food Industries, vol. 7.)

## BEER

This time-honored and popular beverage is one of the class of malt liquors: stout, porter, ale, etc. It is prepared by allowing yeast to ferment hydrolysed starch derived from cereal grains. The hydrolysis is brought about by amylase in the grains which are allowed to *malt*. Malting is necessary since yeast does not produce amylase and, therefore, cannot attack starch as such. The products of hydrolysis are mainly maltose and dextrins. At the same time, proteolytic enzymes convert proteins in the grain to nitrogenous food. An aqueous extract of such grains prepared at just the time when there are maximum maltose, dextrins and protein derivatives, constitutes a rich nutrient medium. Brewers' yeast (*Saccharomyces cerevisiae*) will grow vigorously in such a medium, fermenting the maltose, etc., and producing ethanol and CO<sub>2</sub> under anaerobic conditions.

In beer-making, good, sound grain is steeped for 2 or 3 days to induce sprouting. The sprouts are afterward removed mechanically and the malt grains are dried. They are later crushed or mashed in warm water and soaked. The nutrient aqueous extract, with its amylase, maltose, etc., called *beer wort*, is drained off. It is heated to kill most contaminants. Hops\* are added for color, flavor and aroma. Sometimes roasted malt is added for additional color and flavor. This is used in making *porter*.

After cooling, a pure culture of brewer's yeast ("barm") is added. This is called "pitching." Rival brewers maintain very special strains of yeast for this process! The wort is aerated at first to stimulate *growth* of yeast; anaerobic conditions prevail later on to favor *fermentation*, when CO<sub>2</sub> and ethanol are produced. After fermentation is complete, the beer is clarified and aged ("lagered"†). Then it is carbonated in bottles and sold.

Beer is subject to much the same sort of microbial spoilages as is wine (see below). It was in the study of such spoilages, or "diseases," of beer and wines, that Pasteur first became famous and developed pasteurization to prevent them. He was one of the first industrial microbiologists.

## WINE

Since World War I wine making has become a large industry in the United States. Strictly speaking, wine is fermented grape juice. However, the term is broadly used to include any properly fermented juice of ripe fruits or aqueous extracts of certain selected vegetable products like dandelions, palm shoots, etc. The fermentable juices contain dextrose and fructose in concentrations of from 12 to 30 per cent. In Europe the fermentation is produced mainly by "wild" yeasts; i.e., those brought to the fruits (largely by insects) from a wide variety of sources. Yeasts similar to the species called *Saccharomyces ellipsoideus* are common in such wines.

While other organisms are usually present, the yeasts soon predominate in the fermenting juice, if conditions are suitable for wine making. Tartaric, malic, and other acids, as well as tannin and other substances, including added sulfur dioxide in commercial wine, tend to inhibit growth of many undesirable organisms in the juices.

\* The Hop vine, *Humulus lupulus*, is cultivated for the papery scales of the female flower. These are dried and powdered for use.

† Lager is German for "to be stored"—"to age."

In modern American commercial practice sterilized fruit juices are inoculated with a pure culture of a desirable species of yeast. The preparation and maintenance of the yeast inocula are the special tasks of the microbiologist. The flavor and aroma of the wine depend on: the source and quality of the juice; the yeast or yeasts in the fermentation; the conditions of the fermentation: temperature, time, amount of aeration, subsequent processing, etc.

The inoculated juice is, as in beer-making, at first aerated to promote active and preemptive growth of yeast. Were this to continue, the fermentation would produce only  $\text{CO}_2$  and  $\text{H}_2\text{O}$  from the sugars in the juice. As soon as a good growth of yeast has occurred, the aeration is stopped and the fermentation proceeds anaerobically, so that ethanol, in concentrations of from 10–20% (vol.), is produced. The new wine is placed in large casks to settle, clarify and “ripen.” It may be further treated as indicated in Fig. 44-4.

Spoilage by *Acetobacter*, molds and other aerobic microorganisms may occur if conditions are not anaerobic and the reaction not sufficiently acid. Undesirable facultative and anaerobic bacteria may grow in the later stages if not excluded by cleanliness and suppressed by an initial rapid growth of yeast, or by preliminary sterilization of the grape juice.

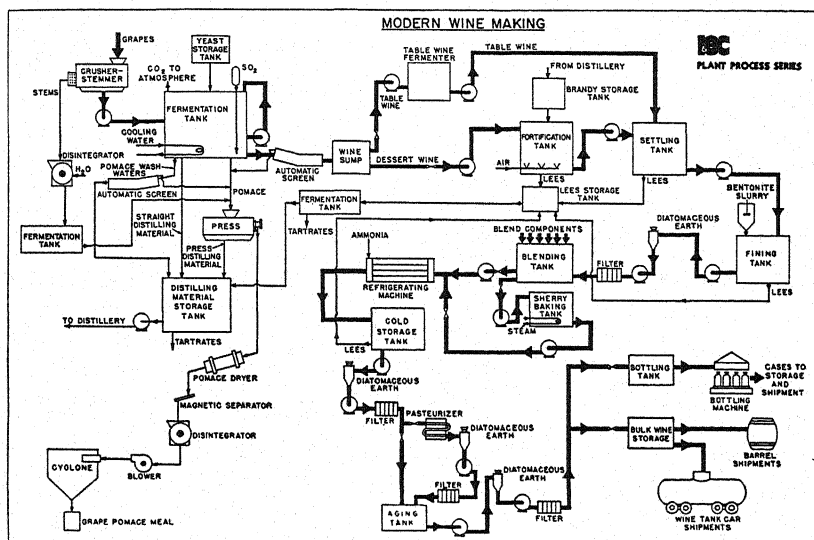


Fig. 44-4. Modern winemaking. At upper left is shown the beginning of the process, with crushing and steaming of the grapes. The juice passes to a fermentation tank, where  $\text{SO}_2$  and yeast are added. White wines are made with juice only. The juice with skins and seeds is made into red wines. During fermentation, temperature is controlled with water coils. After initial fermentation the wine settles and clarifies in a wine sump or tank. The fermented juice is then carried through various processes, depending on the type of wine being made. It may be further fermented for table wine, fortified with brandy for dessert wine, etc. It is later clarified by settling; the lees or sediment being piped off for other uses. The clarified wine is then “fined” by filtration and adsorption with diatomaceous earth and passage through other filters; blended; cooled; clarified; pasteurized; aged and dispensed. These processes differ in mechanical details in every winery. (Hull, W. O., Kite, W. E., and Auerbach, R. C., Ind. and Eng. Chemistry, Oct., 1951, p. 2182. Reprinted by permission.)



## INDUSTRIAL FERMENTATIONS

## TYPES OF PROCESS

In engineering terms industrial fermentation processes may be conveniently divided into 2 main types: (1) the batch fermentation; (2) the continuous process. There are various combinations and modifications of these.

**1. Batch Fermentations.** A tank or fermentor is filled with the ready-prepared mash or other material to be fermented (diluted molasses, comminuted potatoes, digested corn cobs, etc.). The proper adjustments as to pH, temperature, nutritive supplements, and so on are made. Perhaps the mash is steam-sterilized, the entire fermentation tank being the autoclave. The inoculum, a pure culture, is added from a separate, pure-culture apparatus. The fermentation proceeds. Some pressure may be maintained within the tank to prevent inward leakage of contamination and sometimes to maintain increased tension of special gases. After the proper time, the contents of the fermentor are drawn off for further processing, the fermentor is cleaned, and the process begins over again; a discontinuous process divided into batches.

**2. The Continuous Process.** Some fermentations can be conducted as continuous processes. The substrate and inoculum are fed into a container continuously, at a fixed rate. The cells grow (or enzymes act) continuously as the material passes through the apparatus. The product or fully fermented mash is drawn off continuously. The engineering arrangements may be quite complex; permitting aeration, cooling or heating, adjustment of pH, addition of nutrients, etc., continuously during the process.

One may conceive of such a process as taking place in a long pipe (actually it may be a rotating conical tank or series of connected tanks). At one end the raw material, all prepared, enters. It at once encounters the growing organisms. These act on the substrate as it flows through the system. At the stage where the product is at its maximum concentration the fluid is drawn into receiving vessels for further processing (distillation, etc.).

**TWO-PHASE SYSTEMS.** Vinegar manufacture by the generator method is a good example of what is called a two-phase, continuous process. One phase of the system is the alcoholic liquor. It enters the generator at the top and flows downward continuously over the aerated surfaces, to be withdrawn continuously at the bottom as vinegar. The second phase of the system is, of course, the film of *Acetobacter* growing on the surfaces over which the alcoholic liquor trickles.

**Submerged Cultures.** In all processes in which wide-surface, shallow-culture methods are used to secure good aeration, the tendency now is to develop means for tank cultivation with resultant increase in volume of product at lower cost. This type of cultivation is particularly adaptable to continuous-process methods.

Submerged growth during tank cultivation of strictly aerobic organisms necessitates thorough aeration of the contents of the tank by agitation or bubbles of air. This helps remove  $\text{NH}_3$  and  $\text{CO}_2$  from the culture medium as well as to provide oxygen. It has been highly developed in producing antibiotics. Very careful adjustments of O-R potentials, amount of mechanical agitation, ratio of dissolved oxygen to other ingredients in the medium, pH,

etc., are necessary in such processes. This is one of the many fascinating fields for research in industrial microbiology.

### INDUSTRIAL ETHYL ALCOHOL MANUFACTURE

Industrial ethyl alcohol is now made largely from by-products of "cracking" petroleum to make gasoline. However, the manufacture of ethyl alcohol from the fermentation of various carbohydrates, chiefly by yeasts, was formerly an important industry. It still serves to illustrate industrial fermentation processes in general. A formerly much used source of industrial alcohol in the United States was crude molasses. It is easily handled in pumps and tanks and was always available. For use, it requires only to be diluted and the pH adjusted. The pH is adjusted, usually with sulfuric acid, to around 4.5. This is favorable to the yeast and unfavorable to bacteria. Sometimes nutrients may be added, especially a source of nitrogen such as ammonium sulfate and/or ammonium phosphate.

The mash is rather heavily inoculated with an aciduric and alcohol-resistant strain of yeast, the variety depending on the conditions under which the fermentation is to proceed and the exact end products desired. A good strain of *Saccharomyces cerevisiae* is commonly used. The inoculum comes from a large tank of carefully maintained pure culture, previously inoculated from a smaller seed tank, and the latter from a flask or tube of culture in the laboratory.

The inoculum tank or "yeast machine" is aerated since this promotes rapid growth of yeast cells. The maintenance of purity of the inoculum is a responsibility of the microbiologist and woe betide him if some sporeformer, *Lactobacillus*, "wild" yeast or bacteriophage gets in and ruins 100,000 gallons of "mash!" The mash and all of the machinery are generally sterilized before the inoculation and then cooled. The microbiologist is kept busy at every stage of the process, making cultural and microscopic examinations of the water, mash and apparatus to detect and eliminate contamination.

In the batch process, much used for this purpose, fermentation is allowed to continue for about 48 hours at a carefully controlled temperature of about 25° C, until the yeast stops growing due to the concentration of alcohol and other products (Fig. 44-5). Aeration is used at first to promote rapid growth, but anaerobiosis is soon established to promote alcohol accumulation and prevent its oxidation to carbon dioxide and water.

The fermented mash contains the crude alcohol or "high wine," as it is called. This is usually a mixture of ethyl alcohol and a small amount of glycerol with "fusel oil." The last contains amyl, isoamyl, propyl, butyl and other alcohols with acetic, butyric and other acids, as well as various esters. The high wine is driven off from the mash or "beer" by heat and further purified by fractional distillation.

The chemical reactions involved in the fermentation are complex; the principal stages have been indicated previously. The chief constituents of fusel oil are probably derived from the action of the yeasts on amino acids in the mash. The large amounts of carbon dioxide evolved are purified and compressed in tanks or made into "dry ice." Part of this may be used for cooling the vats.

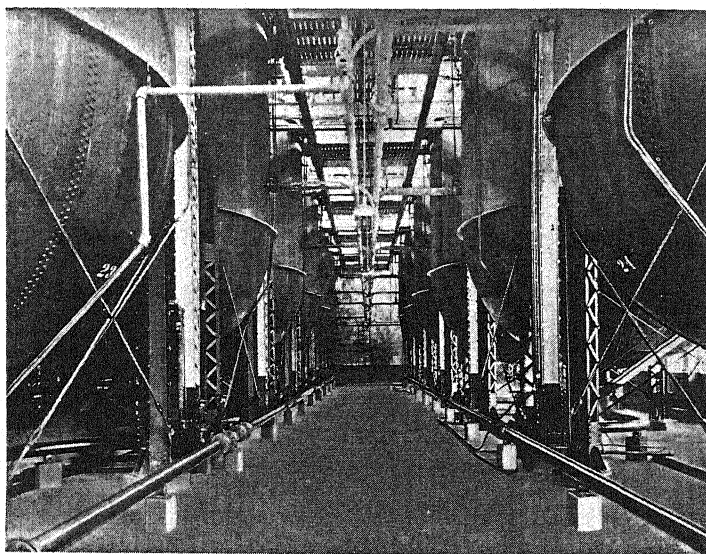


Fig. 44-5. Lower level of 50,000 gallon fermentation tanks. (Commercial Solvents Corp.)

✓ **Wood as a Source of Alcohol and Food.** The utilization of wood by-products ("slabs," sawdust, chips, stumps, etc.), was developed by the U. S. Department of Agriculture. In the Madison Wood Sugar Process the finely chopped wood (cellulose, with lignin, etc.), is treated by allowing 0.6 per cent sulfuric acid to flow slowly over it at a temperature of 150° to 180° F. The resulting liquid contains a variety of fermentable sugars, including glucose, galactose, mannose, xylose and arabinose. The liquid is then brought to a pH of about 5 with lime and put under steam pressure. It is then filtered to remove  $\text{CaSO}_4$  and other insoluble materials.

When cool, nitrogen is added in the form of urea and  $(\text{NH}_4)_2\text{SO}_4$ , the pH is adjusted to about 5.0 and the liquor is inoculated with *Saccharomyces cerevisiae*. Fermentation is allowed to proceed under *anaerobic* conditions, to promote alcoholic fermentation.

**CULTIVATION OF FOOD YEASTS.** In the paper-pulp industry, wood chips are cooked for 6 to 18 hours at 140° F in solutions of calcium bisulfite with free  $\text{SO}_2$ . The waste *sulfite liquor*, after the cooking process, contains much valuable wood sugar and other extractives, forming a good nutrient for *Torula*. By processes similar to those just described these wastes may be turned into masses of yeast by adjustment of pH to about 5.0, removal of  $\text{SO}_2$ , aeration, addition of nitrogen and phosphorus as  $(\text{NH}_4)_2\text{HPO}_4$  and  $\text{NH}_4\text{OH}$ , and inoculation with *Torulopsis utilis*. *Aerobic* growth proceeds in aerated vats. Alcohol is not produced. The separation, drying and pressing of the yeast growth are mechanical details (Fig. 44-6). Yields of up to 50 per cent of the total reducing sugar consumed, in terms of dry torula, are obtainable. These are fed to stock or poultry and so turned into meat and dairy products. Surely the transformation of a knotty old pine slab into a succulent pork chop or a fried egg is modern magic!

**Distilled Beverage Industries.** The production of alcohol for manufactur-

ing and beverage purposes by fermentation and distillation is a valuable and well-developed industry. In principle the production of alcoholic distilled beverages is similar to the production of industrial ethyl alcohol. Refinements are introduced with respect to flavor, aroma, color, sanitation, etc., which are not necessary in the making of industrial alcohol.

There are four general types of distilled liquor: brandy, from fermented fruit juices; rum, from fermented molasses; whiskey, from grains; neutral spirits from mixed grains. In making whiskey and neutral spirits carefully selected grain is stored and ground as needed. Rye whiskey is prepared from mash containing at least 51 per cent rye; bourbon, at least 51 per cent corn. A "spirits mash," intended mainly for the production of alcohol without reference to flavor, i.e., neutral spirits, consists of 88.5 per cent corn, 1.75 per cent rye, and 9.75 per cent barley.

The grain, mixed with water, is autoclaved, cooled, diluted, and 1 per cent barley malt is added to hydrolyze the starch. The "mashing" or hydrolysis,

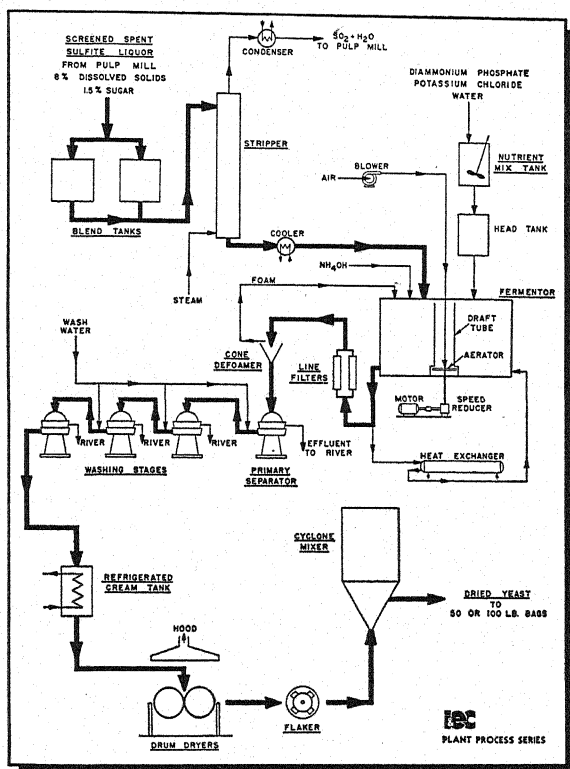


Fig. 44-6. Paper-mill waste to stock-feed. The blended pulp-mill liquors are heated in a "stripper" to drive off  $\text{SO}_2$  and  $\text{H}_2\text{O}$ , cooled and piped to the fermentation tank. Nitrogen and phosphorus are added as ammonium phosphate and as  $\text{NH}_4\text{OH}$ . The yeast culture in the fermentor is aerated to promote maximum growth. The yeast culture is then filtered and passed through a series of centrifuges and other washing and concentrating devices. As "cream" it is finally cooled, dried and packed for shipment. (Inskeep, G. C., Wiley, A. J., Holderby, J. M., and Hughes, L. P. *Ind. and Eng. Chemistry*, Aug., 1951, p. 1702. Reprinted by permission.)

proceeds in a special tank, at around 65° C, for about thirty minutes. The mash is then pumped to the fermentation tanks. Here it is heavily inoculated with selected yeast which has been cultivated in a mash previously made somewhat acid (pH 4.0) with lactobacilli. Fermentation is complete in about seventy-two hours, as in industrial alcohol production.

In the distillation of alcoholic beverages, the fermented mash ("beer") is first distilled *in vacuo*. Aldehydes, esters and fusel oil are then separated from the "high wine" by further fractional vacuum distillation. The details of distillation, blending, etc., do not involve microbiological principles.

The microbiologist maintains the yeast and *Lactobacillus* cultures; carries on research to develop more effective strains; arranges precautions against contamination of the yeast culture vats, fermenters, etc.; makes frequent cultural checks on the various mashes; and examines the bacteria in the air, grain, water supplies, etc. Special investigations and "trouble shooting" are part of his work.

### PRODUCTION OF BUTANOL

As is true of industrial ethanol production, much of our butanol is now derived, as a by-product of gasoline manufacture, from petroleum. However, the biological process is still used to some extent and illustrates important microbiological principles. There are numerous species of *Clostridium* which ferment carbohydrates with the production of butyl alcohol and other materials of value in drugs, paints, synthetic rubber, explosives, plastics, etc. Some species produce isopropyl alcohol and acetone as well. Important among these organisms are *Cl. acetobutylicum* and *Cl. felsineum*. As in the production of acetone and ethyl alcohol, the production of butyl alcohol involves the preparation of mashes from sugars, starches or cellulosic materials by appropriate processes. Complete sterilization of all apparatus is essential because many undesirable bacteria of the environment can grow well under the conditions of the fermentation. Conditions cannot be kept as acid as they are in yeast fermentations because *Clostridium* has its optimum pH around 7.2. Particularly troublesome contaminants are species of *Lactobacillus*. An organism called *B. volutans*, a gram-positive, non-sporeforming rod, (possibly a species of *Lactobacillus*?) is also especially dangerous.

Fermentation proceeds anaerobically for about three days. Normally butyl alcohol, acetone and ethyl alcohol, with carbon dioxide and hydrogen in large amounts, predominate when *Cl. acetobutylicum* acts in a glucose mash. Other substances may occur in smaller amounts. Riboflavin (a vitamin of the B complex) is a valuable constituent of the residue after distillation of the solvents. Butyl and isopropyl ("rubbing") alcohols are important among the fermentation products of *Cl. butyricum*.

**Relation of Sporulation.** The most vigorous and productive fermentations by species of *Clostridium* are brought about by cultures of the organisms which have undergone a series of heat treatments ("heat shocking") to select vigorous and rapidly sporeforming cells. A long process of alternate heatings to kill vegetative cells, and periods of vegetation and sporulation which are checked abruptly by heating again, select the most rapid and resistant sporulating survivors.

## OTHER PROCESSES: USE OF "BY-PRODUCTS"

A number of other valuable organic substances widely useful in medicine, industry, etc., are produced by analogous processes or as by-products. Among these are lactic acid, produced from crude carbohydrate mashers by *Lactobacillus* or *Streptococcus lactis*; ethylene glycol formed during alcoholic fermentations; *butane diol*, used in the manufacture of synthetic rubber, a by-product of industrial alcohol manufactured by fermentation, and also produced from dextrose by bacteria such as *Aerobacter*; *citric acid*, produced from hexose by molds of the genera *Aspergillus* and *Penicillium*; *oxalic acid*, also produced from hexose by *Aspergillus* species; *gluconic acid*, produced from glucose by *Penicillium chrysogenum*, one of the molds valuable in the production of penicillin.

Waste products such as sawdust, shavings, garbage, beet-pulp, horse chestnuts, rice, peat moss, waste molasses, and, in fact, almost any fermentable or putrescible substance, can be made to yield valuable products by treating them with the proper microorganisms under controlled conditions. The possibilities are limited only by the extent of man's knowledge of microorganisms and his ingenuity in making use of them. Even combustible gases consisting of methane and hydrogen are made from sewage and are used for heat and power on the premises of disposal plants. The microbial processes cannot at present compete with natural or coal gas for municipal or industrial use.

## ENZYMES OF MICROORGANISMS IN INDUSTRY

As already indicated, in a number of industrial fermentation processes the amylase of malt is used to "convert" or hydrolyze the starch in grain kernels so that it may be fermented by yeast or bacteria. Shortages of grain malt have directed attention to other sources of amylases. A long-known source of amylases is the group of molds. Several processes for producing mold enzymes have been developed. In the modified "Amylo" process a mash is made with flour. The flour mash receives an inoculum of pure culture of yeast and is immediately mixed with a pure culture of mold. The amylases of the mold hydrolyze the flour starches completely and the yeast ferments the hydrolyzed starch. The alcohol is distilled off in the usual manner.

## ENZYMES FROM BACTERIA

**Amylases and Proteases.** Both amylolytic and proteolytic enzymes are produced in sufficient quantity by certain bacteria (notably species of *Bacillus* like *B. mesentericus*, *B. macerans* and *B. polymyxa*) to be of commercial value. The amylolytic enzyme obtained from these organisms is used in the brewing and related industries to convert raw starches into materials fermentable by yeasts. These enzymes are also much used in the textile and paper industries.

**PRODUCTION.** Amylolytic and proteolytic enzymes from *B. mesentericus* are prepared in culture medium made from various organic wastes: hydrolyzed casein, soybean and peanut cake, dextrins, etc., with suitable nutrient supplements. The medium is first sterilized by autoclaving. It is then inoculated with a selected strain of the organism to be used. After about a week the mash is centrifuged at high speed and the clarified fluid is collected and

stored at low temperature or preserved with disinfectants. It may be concentrated by low-temperature dehydration through distillation in vacuo.

### MICROBIOLOGICAL ASSAYS ✕

It has already been explained (Chapter 15) that, while some organisms may synthesize, from simple inorganic materials, amino acids, vitamins and other complex substances essential to their growth, other species are deficient in this respect. In a medium complete in all nutrients except one of these essential factors, growth of the nutritionally deficient species will not occur. The auxotrophic mutant exemplifies this condition. If a minute amount (say 0.01 gamma per ml) of the essential factor be added, some growth will occur. More growth will occur in the presence of more of the missing factor. Up to the point of satiation, growth bears a linear relationship to the amount of the specific growth factor added. Let us suppose that the growth factor (say nicotinic acid) has been added in graded amounts to a series of tubes of such a deficient culture medium, not as pure nicotinic acid but in the form of some food substance, say an extract of fresh green beans. Accurate, photometric measurements are then made of the growths obtained in the series of cultures. By comparing them with growths obtained in a parallel series of tubes of the same medium containing pure nicotinic acid in graded, known concentrations, it is possible to estimate closely the concentration of the growth factor in the green beans. This method of estimation of growth factors in foods is spoken of as *microbiological assay*.

Certain organisms lend themselves very well to such assay procedures. *Lactobacillus casei* and *L. arabinosus* are easy to cultivate, relatively hardy, harmless, and unable to synthesize several growth-factors including various amino acids, riboflavin, biotin, pantothenic acid, nicotinic acid, etc. Other organisms may be used for assay of other substances: for example, yeast for thiamine; *Streptococcus lactis* for folic acid; *Neurospora sitophila* (a pyridoxine-deficient ["pyridoxineless"], ultraviolet-induced, auxotroph) for pyridoxine; and so on.

### ASSAY METHODS

The basic principle of all of the assays is the same. Four illustrative methods of measuring growth of the test organisms are:

1. **Measurement of gas (CO<sub>2</sub>)** produced during fermentation of sugar in the test culture. Yeast is routinely used in the microbiological assay of thiamine by this method.

2. **Measurement of acid production by bacteria** from glucose in the test medium. Lactobacilli are commonly used in assays of nicotinic acid, riboflavin, etc., by this method.

3. **Measurement of turbidity** due to bacterial growth, using a photoelectric turbidimeter. This method may be used with *Lactobacillus*, or with *S. lactis* in folic-acid assay, or other assays.

4. **Gravimetric measurement of the mycelia of molds** like *Neurospora* after removal from the test culture and drying. This procedure is used in pyridoxine assays.

Microbiological assays are strongly affected by various factors which influence the test organisms. For example, *aerobically*, *L. lactis* will die before

it will grow without vitamin B<sub>12</sub>. *Anaerobically*, it *sneers* at vitamin B<sub>12</sub>! There are many other examples. We may smile, but knowledge of such peculiarities is essential to successful assay procedures.

### INDUSTRIAL SPOILAGE

In contrast with the useful activities of bacteria, a word may be said of their destructive action. Several causes of industrial spoilage (e.g., "diseases" of fermentations) have been mentioned in this chapter and in the chapters on soil, food, and water bacteria. Species of *Micrococcus*, *Alkaligenes*, *Flavobacterium*, *Serratia*, *Clostridium*, coliform organisms, yeasts, molds, etc., are common causes of spoilage. Each type of product is attacked by certain species of microorganisms which can metabolize the substance especially well. For example, spoilage of cellulosic products, such as wood, paper, fibers, tobacco, cotton, etc., is brought about by cellulose decomposers like molds, *Cellulomonas*, *Cytophaga* and anaerobic organisms of the soil. Fermentable substances like syrups, beverages, etc., are attacked by yeasts, lactobacilli, organisms of the coli-aerogenes group and various environmental bacteria including the genus *Clostridium*. Spoilage of proteins like meats, fish, milk and so on results from the action of proteolytic species such as *Pseudomonas*, *Bacillus*, *Proteus*, *Micrococcus*, *Clostridium* and many others. Rubber and petroleum are attacked by certain bacteria.

*Lactobacilli* and *Leuconostoc* have already been noted as particular villains in the acid food, fermentation and distillery industries. For example, species of both ruin thousands of dollars' worth of orange juice annually during concentration. They produce a "buttermilk" flavor. Pasteur found lactobacilli and *Leuconostoc* causing "diseases" of beers and wines. They are just as active today. Lactobacilli also discolor meats, especially producing greenish discoloration (oxidized porphyrins) of cured hams and sausages. The slimy dextran- or levan-forming species, like *Leuconostoc mesenteroides* and *L. dextranicum* and some lactobacilli, produce slimy and "ropy" conditions in a great variety of human endeavors: sugar refineries, pickle brines, dairy products, ham-curing cellars, and the like. These organisms prefer acidified products such as partly fermented foods, "mashes," citrus juice, etc.

Development of undesirable flavors in butter, especially rancidity, is due in great part to the formation of butyric acid as a result of lipolysis. It is due to species of *Aspergillus* and other molds, *Pseudomonas* species and streptococci related to *Str. liquefaciens*. These difficulties do not arise when clean equipment, clean milk and proper precautions are used.

Proteolytic organisms, like *Str. liquefaciens*, are responsible for undesirable bitter flavors and early spoilage of cheeses. Gas production is usually due to coliforms and/or *Clostridium*; putrefaction or digestion to *Clostridium*, *Pseudomonas*, *Bacillus*, etc. Such conditions result mainly from dirty milk or equipment or careless handling.

**Prevention of spoilage** depends on maintaining conditions unfavorable to, or excluding by asepsis, organisms which can grow on or in the particular product involved. This may involve complete sterilization, drying, refrigeration, aeration, the use of inhibitory salt, sugar or acid concentrations, radiation with ultraviolet light, exposure to sunlight, treatment with substances like creosote, sodium benzoate and the like. Each type of spoilage is a problem



in itself and research in such fields, as well as in the field of productive microbiology, has proven interesting and lucrative to those sufficiently interested to make a special study of the matter. A thorough knowledge of selective bacteriostatic methods for specific types of organisms is valuable in this field.

## REFERENCES

- Barton-Wright, E. C.: *The Microbiological Assay of the Vitamin B-Complex and Amino Acids*. Pitman Publishing Corp., New York, 1952.
- Bennett, F. W., and Nelson, F. E.: Action of aerosols of certain viricidal agents on lactic streptococcus bacteriophage. *J. Dairy Sci.*, 1954, 37:840.
- Bunker, H. L.: *Microbes and History*. The "acetone bacillus." *Lab'y. Pract.* (London), 1957, 6:36.
- Cheese Varieties and Descriptions: U. S. Dep't of Agr. Handbook No. 54, 1953. Sup't of Doc., Washington 25, D. C.
- Collins, E. B.: Action of bacteriophage on mixed strain cultures. III-IV. *Appl. Micr.*, 1955, 3:137, 141, 145.
- Czulak, J., and Naylor, J.: Host-phage relationships of cheese starter organisms. I, II, III. *J. Dairy Res.* (London), 1956, 23:No. 1.
- Eckles, C. H., Combs, W. B., and Macy, H.: *Milk and Milk Products*. McGraw-Hill Book Co., Inc., New York, 1951.
- Etchells, J. L., Bell, T. A., and Jones, I. D.: Morphology and pigmentation of certain yeasts from brines and the cucumber plant. *Farlowia*, 1953, 4:265.
- Gavin, J.: Analytical microbiology, I. The test organism. *Appl. Micr.*, 1956, 4:323.
- Greathouse, G. A., Wessel, C. J., and Shirk, H. G.: Microbial deterioration of manufactured materials. *Ann. Rev. Microbiol.*, 1951, 5:164.
- Hajny, G. J., et al.: Thermophilic fermentation of cellulosic and ligno-cellulosic materials. *Ind. & Eng. Chem.*, 1951, 43:1384.
- Hull, W. A., Kite, W. E., and Auerbach, R. C.: Modern winemaking. *Ind. & Eng. Chem.*, 1951, 43:2180.
- Jezeski, J. J.: The microbiology of dairy products. *Ann. Rev. Microbiol.*, 1954, 8:429.
- Ledingham, G. A.: Industrial fermentations. *Ann. Rev. Microbiol.*, 1953, 7:443.
- LeMense, E. H., Corman, L., Van Lanen, J. M., and Langlykke, A. F.: Production of mold amylases in submerged culture. *J. Bact.*, 1947, 54:149.
- Maxon, W. D.: Microbiological process report: Continuous fermentation. *Appl. Micr.*, 1955, 3:110.
- Perret, C. J.: An apparatus for the continuous culture of bacteria at constant population density. *J. Gen. Micr.*, 1957, 16:250.
- Prescott, S. C., and Dunn, C. G.: *Industrial Microbiology*. 2nd ed. McGraw-Hill Book Co., New York, 1949.
- Richards, T.: Spoilage of industrial materials by microorganisms. *Nature*, 1954, 173:102.
- Shearon, W. H., and Weissler, H. E.: Modern brewing. *Ind. & Eng. Chem.*, 1951, 43:1262.
- Smith, G.: *An Introduction to Industrial Mycology*. E. Arnold & Co., Ltd., London, 1946.
- Thatcher, F. S.: Foods and feeds from fungi. *Ann. Rev. Microbiol.*, 1954, 8:449.
- Underkoffler, L. A., and Hickey, R. J.: *Industrial Fermentations*. Chemical Pub. Co., New York, 1954.
- Various Authors: Studies of microbiological spoilage, or damage to: electrical insulating materials, fire hose, paper and paperboard, vulcanized rubber. *Appl. Micr.*, 1955, 3:75, 82, 86, 89, 302; Coffee, Milk, Orange Juice. *Ibid.*, 1956, 4:69, 97.
- Wedberg, S. E.: *Microbes and You*. The Macmillan Co., New York, 1954.
- White, J.: *Yeast Technology*. John Wiley and Sons Co., New York City, 1954.
- Wright, L. D.: Nutrition of bacteria and fungi. *Ann. Rev. Microbiol.*, 1956, 10:141.

## Some Viral Diseases

**Virus Groups.** For convenience we may arrange viruses infecting human beings into at least six groups (Table 31), each group designated according to the tissues of the body with which the viruses in the group appear to be most obviously associated.

It must be clearly understood that, while this arrangement is often used, it is not an exact, established, or professionally accepted grouping. It is merely a usage of convenience and "subject to change without notice." Some of its inaccuracies will become apparent in the discussion.

### A VISCEROTROPIC VIRUS

One of the viral diseases of man and animals which has been much investigated is yellow fever. Yellow fever virus was the first viral agent of human disease to be discovered. The facts revealed by those who investigated yellow fever and yellow fever virus served to point the way to investigators of other viruses. In addition, the history of yellow fever is of some interest.

**Yellow Fever.** This is often cited as an example of a viscerotropic virus because the normal form of the virus affects chiefly the liver and other viscera. Yellow fever is now endemic\* only in certain tropical regions. The disease was probably brought from Africa to the Western Hemisphere by Portuguese and Spanish explorers and slave traders as early as the year 1500. It was later distributed through all the Caribbean Islands, our own Gulf states and Atlantic seaports and Central and South America, taking a terrific toll of life wherever it appeared. For centuries the "yellow jack" as it was called, made life in the tropics a matter of extreme peril. No one knew how it spread or how to avoid it. Hindle states, "In the 90's of the last century the crews of ships going to South and Central American ports were to a large extent shanghaied (kidnapped and forced into service) and this captain himself obtained command at the early age of twenty-one by volunteering to take the ship to Santos. On this particular voyage all except three of a crew of twenty-one died of yellow fever, so their fears were fully realized." When the French tried to build a canal through Panama, the laborers died in such numbers from yellow fever, malaria and dysentery, that the attempt was abandoned.

\* Constantly present among the human inhabitants.

During the Spanish-American War, many hundreds of American soldiers died of the disease in Cuba, even after a general campaign of "sanitation" had been carried out.

The military authorities finally appointed a yellow fever commission composed of James Carroll, Jesse W. Lazear and Aristides Agramonte under the direction of Walter Reed. These men set out, in 1899, with a grim determination to solve the problem of yellow fever.

Many different kinds of bacteria had been blamed as the cause. Reed first made a thorough bacteriological investigation, testing each by Koch's postulates, and came to the conclusion that the disease was not due to bacterial infection.

**Table 31.** *Apparent Tissue Tropisms of Some Mammalian Viruses.*

TISSUE GROUPS	REPRESENTATIVE VIRUSES
<i>Viscerotropic</i> Associated with the viscera, especially the liver	Homologous serum jaundice, yellow fever
<i>Respiratory</i> Associated with respiratory tract	Influenza, adeno viruses
<i>Dermotropic</i> Associated with skin and mucous membranes	Smallpox, measles, German measles
<i>Neurotropic</i> Associated with nervous tissues	Poliomyelitis, rabies, encephalomyelitis*
<i>Enteric</i> Associated with gastrointestinal tract	Poliomyelitis, diarrhea of newborn, ECHO viruses
<i>Neoplastic</i> Associated with cancers	Warts, animal and plant tumors

\* *Encephalon* is Greek for brain; *myel* is from the Greek for a central core; the spinal cord. Hence, encephalomyelitis = inflammation of brain and spinal cord.

A generally held idea was that fomites contaminated with the vomitus or feces of victims of yellow fever were very dangerous vectors of the disease-producing agent, whatever it might be.

Another idea, advanced by Carlos Finlay, a Cuban scientist of Scottish descent, was that yellow fever was transmitted by the bite of *Aedes aegypti* mosquitoes. Finlay's idea was treated rather contemptuously by many wise men of the times. It seemed preposterous!

Walter Reed and his colleagues arranged experiments to test these two hypotheses. Near Havana they built a little, screened house, divided into two halves. They put beds, chairs and other simple furnishings on each side. In one compartment they piled blankets, sheets, bedding, clothes, and other fomites of men who had died of yellow fever. No mosquitoes were allowed in this

compartment, but a number of the insects, known to have bitten yellow fever patients, were placed in the other compartment which was clean and free from fomites.

Since Reed and his co-workers had not found any animal which was susceptible to the disease, and which they could use for the experiment, human volunteers were asked to live in the compartments for days; to be imprisoned with lurking, flying death; to sleep in the beds; live in the clothes and eat from the dishes soiled by men who had died of yellow fever. It was not a pleasant invitation but there were numerous volunteers among the American troops! For days nothing happened. Then, one after another, men in the mosquito compartment became ill with yellow fever. Fortunately none of the men died. The men in the other compartment remained well! The secret was at last wrrenched from nature! The mysterious messenger of death was at last revealed as a certain tropical mosquito (*Aedes aegypti*). Meanwhile Lazear, allowing himself to be bitten in a yellow fever hospital by an infected mosquito, lost his own life. An Army nurse, Clara Louise Maass, experimenting with active immunization against the disease in the same manner, likewise perished. Carlos Finlay's "ridiculous ideas" were fully verified.

Although the means by which yellow fever was *transmitted* was known, the actual *cause* of the disease was not. Walter Reed tested the filtrability of the infectious agent in the blood of yellow fever patients and easily proved that the causative agent was not an ordinary bacterium but an *invisible, non-cultivable, filter-passing* virus.

It was found that the virus circulates in enormous amounts in the blood of the patient but only during the first 3 to 5 days of the disease. It is only during this period that a biting mosquito (only female mosquitoes bite!) can acquire the virus. Further, a period of 8 to 14 days (depending on climatic temperatures) must elapse during which the virus multiplies in the mosquito and/or migrates from stomach to salivary gland. The mosquito's *bite* is not infectious during this period of *extrinsic incubation*. However, the virus is present in an infectious stage during the period, as may be proven by grinding the mosquito in a little serum and injecting it into a susceptible animal. The mosquito remains infectious during her life (about 90 days). Yellow fever is a good example of an *arthropod-borne, viscerotropic* virus. Others are dengue and Rift-Valley fever.\*

It is evident that yellow fever may attack susceptible populations in areas where *Aedes aegypti* are numerous. These are always cities since *A. aegypti* does not breed in jungles but only in *artificial, domestic*, water containers. Epidemics have occurred during summer months in many northern seaports, the mosquitoes and virus being carried from the tropics by ships. The danger of airplane-borne yellow fever is obvious, since many places have large numbers of *Aedes aegypti*, including India and our Gulf states.

The remainder of the story is one of confirmation of Reed's work, study of the virus, campaigns against *Aedes* mosquitoes and production of an effective vaccine. Yellow fever disappeared from Cuba! The Panama Canal became a

\* There is also a large group of *neurotropic* viral diseases which are arthropod-borne, notably the encephalomyelitides like St. Louis encephalitis, Japanese B fever, Eastern equine encephalomyelitis, and many others. These affect the central nervous system, producing various nervous disturbances including what is called "sleeping sickness."

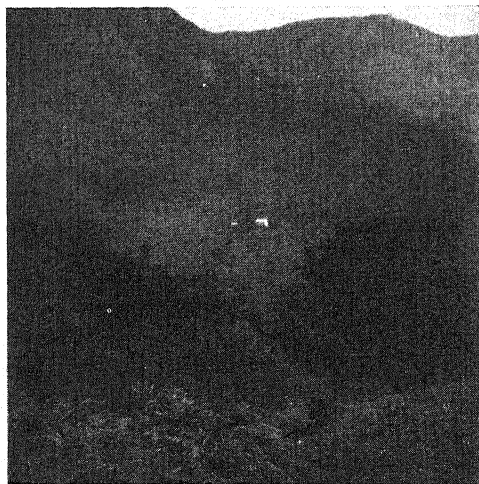


Fig. 45-1. One of the first houses where jungle yellow fever was discovered. Remembering that yellow fever had for centuries been considered a disease strictly of populous cities it was surprising as well as enlightening to find it in such remote habitations. This is in the Valle de Chanaan, Espirito Santo, Brazil.

practical project. The islands of the Caribbean became more healthy, and the "yellow jack" retreated, snarling, as it were, to the interior of South America. The International Health Division of the Rockefeller Foundation, under Russell and Sawyer, carried on the war against it.

**JUNGLE YELLOW FEVER.** In 1932 Soper, Cardoso, Seraphim, Frobisher and Pinheiro made the discovery that yellow fever persists in remote parts of the wooded and rural portions of tropical South America (Fig. 45-1), where it is transmitted by jungle mosquitoes (*Hemagogus spegazzinii* and others) and is maintained there among wild animals, especially monkeys; notably howler monkeys (*Alouatta*) and spider monkeys (*Ateles*). It is referred to as "jungle yellow fever." A parallel situation (with different species of monkeys and mosquitoes) was found to exist in Africa. Jungle yellow fever is readily transmitted from monkeys to man when the latter visits the jungle. The infected man can carry the virus home to the city where, if *Aedes aegypti* are prevalent, he can start an urban outbreak of classical yellow fever.

In 1956 jungle yellow fever spread (mainly in howler and spider monkeys) to Panama, Costa Rica, Nicaragua, Trinidad, Honduras, and Guatemala. There is every reason to anticipate its appearance in Mexico. Will it cross the border into California, Arizona, New Mexico or Texas; or be transmitted to our *Aedes*-infected gulf coast by plane or auto?

**CONTROL OF YELLOW FEVER.** Yellow fever is controlled by two principal procedures: (1) eradication of *Aedes aegypti*; (2) vaccination. Mosquito control in cities is a relatively simple matter of administration and entomological engineering. In jungles it is impossible. Vaccination is necessary.)

*Yellow fever vaccine* was developed in the decades beginning in 1930, as a culmination of intensive research in Europe, Africa and North and South America, costing the lives of many devoted scientists; affording honors and fame for some, death for others; the way of life!

First it was found, by Stokes, Bauer and Hudson, that *rhesus* monkeys were as susceptible as human beings, thus eliminating the necessity for human volunteers. Later Theiler (Nobel prize) et al. found that the virus could be modified to a very virulent *neurotropic* type (selection of a mutant?) by continued injection and reinjection into the brains of mice. In this neurotropic state it would not produce typical yellow fever if inoculated into skin or muscle but would *immunize!* Later, this virus was modified still further by propagation in tissue-culture, using both chick tissues and those of embryo mice, as well as live chick embryos in eggs. After many trials the neurotropic virulence and also the viscerotropic virulence were reduced to the point where the virus produced no disease but did immunize solidly. This was culture No. 17D, from which the now famous, live, attenuated, yellow-fever vaccine was derived. The nature of the mutation which resulted in the harmless 17D strain is still unknown.

The vaccine as used consists of chick embryos containing the 17D virus (Fig. 45-2). These are minced, ground, clarified, diluted with serum-saline fluid, and thoroughly tested for antigenicity, absence of bacteria, and harmlessness. It is one of the most effective agents of artificial immunization.

#### RESPIRATORY VIRUSES

There is a large (and growing) family of viruses which cause acute infections of the respiratory tract. One of these is the well-known, clearly differentiated, often fatal disease called *ornithosis* or *psittacosis*\* ("parrot fever"), caused by one of the large-type, antibiotic-sensitive viruses and transmitted by infected

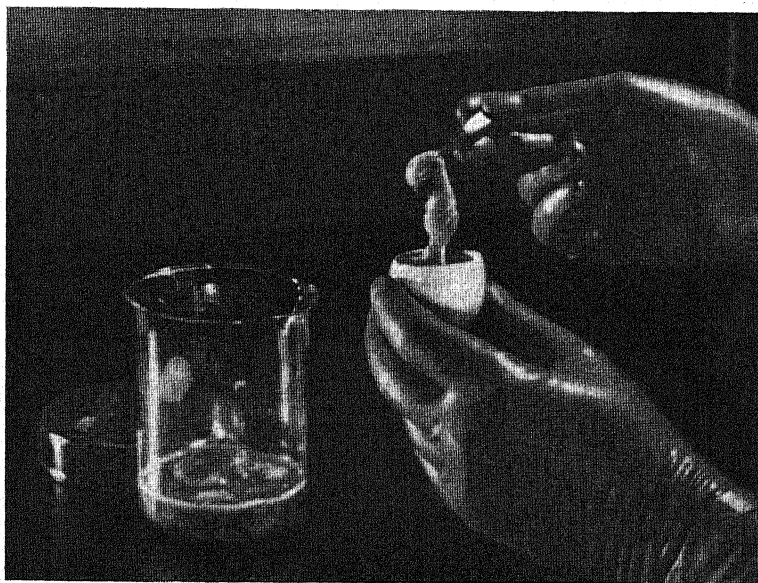


Fig. 45-2. The infected chick embryo and membranes (above) containing virus are removed from the egg under aseptic conditions. (Therapeutic Notes, Parke, Davis & Co.)

\* The name is derived from the Latin word for parrots, love-birds etc.: *psittacus*.

birds of many species. The disease affects many species of birds and is often called ornithosis. Another well-defined and too-familiar respiratory infection is *influenza*. A less clearly delineated entity is called *primary atypical pneumonia*, supposedly due to a definite virus but possibly a result of infection with one of several similar viruses. Then there is a group of still less clearly-defined, probably closely related viruses causing conditions formerly classed as "influenza-like," the "common cold" or "cold-like," formerly referred to as *acute respiratory disease* (ARD), *respiratory illness* (RI), and *adenoidal-pharyngeal-conjunctival* (APC) fever, or *adenoidal degeneration* (AD). These designations were based on distinct clinical and epidemiological characteristics of the diseases they referred to.

The relationship of these viruses is still not clear. In brief, it may be said that there appears to be one more or less distinct group of viruses associated with influenza-like and pneumonia-like conditions (RI and ARD) and another associated with the catarrhal-cold syndromes (APC and AD and non-bacterial pharyngitis). All of these viruses are now grouped as *adenoviruses*.

✓ **Human Influenza.** This virus is representative of respiratory viruses in general. It enters and leaves the body via the oral and nasal tracts. The symptoms of influenza are often confused with those of other acute respiratory diseases, and many other diseases as well. Influenza is generally a markedly febrile disease, extremely weakening, accompanied by muscular pains, sweating, and sometimes by symptoms of the common cold as well. Recovery is often delayed for weeks and fatality is sometimes high, as in the pandemic of 1918-1919. It is particularly apt to be fatal in patients with other chronic diseases: heart, diabetes, etc., and during pregnancy. ✓

**TYPES OF HUMAN INFLUENZA.** At least three main types of epidemic influenza virus have been discovered: A, B, and C. These viruses, while causing clinically indistinguishable diseases, and although very much alike, are entirely distinct immunologically. Other varieties of influenza virus, some quite distinct and some more or less closely related to A, B, and C, have been described (A', etc.). These strain differences are of the greatest importance in respect to the preparation of an effective vaccine, as vaccination against some strains gives no protection whatever against certain others.

Influenza viruses appear to mutate or vary in antigenic and immunologic properties very readily, both under conditions of laboratory manipulation and in nature. It appears that new and distinct antigenic variants are appearing much faster than our ability to prepare vaccines against them.

The type of epidemic influenza which occurred in highly lethal form in the 1918-1919 world-wide epidemic may have been due to still another form of the virus, which is sometimes differentiated as "the virus of *pandemic influenza*." The true etiology of the 1918-1919 epidemic is still a matter of surmise.

All of the known viruses of human influenza can be propagated in chick embryos from which vaccines may be prepared.

The importance of research on these diseases, especially on the possibility of artificial immunization, is obvious in view of the estimated loss of over 100,000,000 man-days of work annually due to these diseases and the expenditure of over \$7,000,000 by the public annually on remedies which do not cure any of them. All this and misery too!

## DERMOTROPIC VIRUSES

**Measles.** Measles, generally classed with the dermatropic viruses, is so common that little description is needed though a clinical diagnosis is sometimes not easy. It is believed that susceptibility of man to measles is universal, except in infants up to about 6 months whose mothers were immune (natural, passive immunity). Natural active immunity is high grade and life-long. The disease is entirely distinct from German measles.

The early symptoms of measles are much like those of a cold. The oral and nasal secretions in early stages are highly infective. Thus, measles might be considered as due to a respiratory virus. During this stage measles is probably the most readily transmitted of all infectious diseases. ✓

*Temporary passive protection* by means of antibodies (gamma globulin) from the serum of immune persons is used to prevent the development of measles in children under about five years of age. In young children the disease is most likely to be followed by pneumonia and other dangerous complications frequently fatal. In older children the dosage of gamma globulin may be reduced so that a mild, immunizing attack is permitted to occur in childhood. This prevents a possibly costly, and certainly inconvenient, lay-up during adult life. Work on tissue-culture of viruses from measles patients gives promise of a vaccine similar to polio vaccine in some respects.

Another important but now rare (in the U.S.) dermatropic disease is small-pox. It has been discussed in connection with immunization (Chapters 21, 22).

## PRENATAL INJURY BY INFECTIOUS AGENTS

A number of bacterial infections, notably syphilis and typhoid fever, occurring in women *during the first 3 or 4 months of pregnancy* can cause serious damage to the embryonic tissues.

Among the viral diseases, influenza was seen to have a disastrous effect on pregnant women and unborn children in the great pandemic of 1918. About half of the expectant mothers died. Of the survivors about 60 per cent had abortions or lost the children at birth or soon after.

**Rubella and Pre-natal Injury.** In 1941 Gregg, in Australia, noted that children born of mothers who contracted rubella (German measles) during the first three months of pregnancy frequently had defects of hearing and eyesight or deformities of the heart, feeble-mindedness, and other injuries. Confirmatory data were collected elsewhere, including the United States. Rubella, previously generally regarded as of little consequence, is caused by a virus which has a predilection for *early embryonic* tissues upon which it exerts a destructive effect; hence the various malformations mentioned above. In the absence of effective means of vaccination some physicians recommend exposure of girls to the disease between the ages of 6 to 8 years so that they may become immune and thus not contract the disease during pregnancy later in life. Non-immune mothers may be protected during early pregnancy by passive immunization with an antibody-bearing fraction (gamma-globulin) of the blood of immune persons.

## NEUROTROPIC VIRUSES

**Poliomyelitis.** Infantile paralysis or, more properly, anterior poliomyelitis is an acute, febrile, viral disease, world-wide in distribution, and most com-



mon (75 to 80%) in children 15 years and under, but not confined to childhood. Typical cases are characterized, as are many viral diseases, by sudden onset and a febrile attack with nausea, headaches, sometimes stiff neck, and muscular pains. In the vast majority of infections with the virus (which includes virtually everyone) the disease is short and mild; usually passing unnoticed, unrecognized, or confused with influenza. Complete recovery and presumably life-long immunity are the rule. In a relatively small percentage of cases the disease is severe. The principal and characteristic damage is to nervous tissues, especially to those nerves which are in association with the muscles. Muscular paralysis with resulting degeneration sometimes occurs.

Poliomyelitis viruses are among the smallest (10–15  $\mu$ ). There are several immunologically different varieties of the virus, so that we may well use the plural form: poliomyelitis viruses. At least 3 types of these viruses: I, II and III are known. Infection with a polio virus of any one of these types confers immunity to polio virus of that type but not other types. The disease is type-specific, like influenza.

**POLIO VIRUS IN THE BODY.** Poliomyelitis virus probably multiplies primarily and commonly in the tissues of the upper respiratory and gastrointestinal tracts. Polio virus of one or another type is constantly present in the gastrointestinal tract of a very considerable proportion of the population, wholly without their knowledge; being transmitted continually from individual to individual. Clearly, carriers are very important. The virus is common in normal feces and in sewage. Only when it invades nervous tissues fairly extensively does the recognizable form of the disease occur. It is sometimes present in the blood stream. As in many other maladies there are a great many infections, yet only occasionally (about 1%) does recognizable disease occur. The virus of poliomyelitis appears to gain entry to the body through the intestinal tract and/or the upper respiratory tract. Thus, it has neurotropic, enteric, respiratory and viscerotropic aspects (Fig. 45-3).

**TRANSMISSION OF POLIO.** The virus leaves the body in the feces and (during the early stages of the disease) in oral and nasal secretions. Apparently animals do not harbor the virus in nature, nor is there any evidence that house flies, biting insects, pasteurized milk or properly chlorinated drinking water are of importance in transmitting the disease. It appears to be transmitted, in the vast majority of instances, by means of feces and articles (HANDS!) soiled with feces. The great prevalence of the virus (and of immunity to it) is an indication of how frequently we (unknowingly) are subject to oral-fecal contamination. Nearly all urban adults have immunity to poliomyelitis of one or more types.

**IMMUNITY IN POLIOMYELITIS.** Infants born of immune mothers have passive immunity for a few months but soon become susceptible. If exposed to a known case they may receive some passive protection by promptly receiving gamma globulin from the serum of an immune person. The protection thus given is far from perfect and, at best, temporary. Active immunization is much better.

**THE SALK VACCINE.** The so-called Salk vaccine consists of poliomyelitis virus (three immunologic types) cultivated in living cells of monkey kidney tissue while growing in a complex nutrient fluid in flasks. The growth of the virus results in injury and death of the kidney-tissue cells. These changes can

be recognized and are observed daily by microscopic examination of the culture. After appropriate incubation (several days) the dead and injured tissue cells and detritus are removed by filtration. Formalin is added to a concentration of 1:4000 and the vaccine fluid is allowed to stand for about a week or until tests show that all of the virus is inactive. After appropriate tests for sterility and potency, and after removal of the formaldehyde and addition of a mercurial preservative, the vaccine is ready for use. Modifications and improvements in the process are being constantly made.

Like diphtheria toxoid, two preliminary doses of the vaccine are given, about four weeks apart, followed by a "booster" dose about seven to eight months later. Untoward reactions to the vaccine are no more severe or frequent than to other types of immunizing agents commonly in use.

Large-scale trial of the vaccine, the results of which were announced to the public by world-wide press and radio facilities on April 12, 1955, showed that, properly prepared, the vaccine is: (1) *safe* and (2) *effective* to a satisfactory degree. It does not necessarily prevent primary infections of the alimentary tract. Such infections in themselves do relatively little harm and serve to stimulate protective antibody production. The vaccine does, however, call forth enough antibodies to prevent passage of the virus from the alimentary

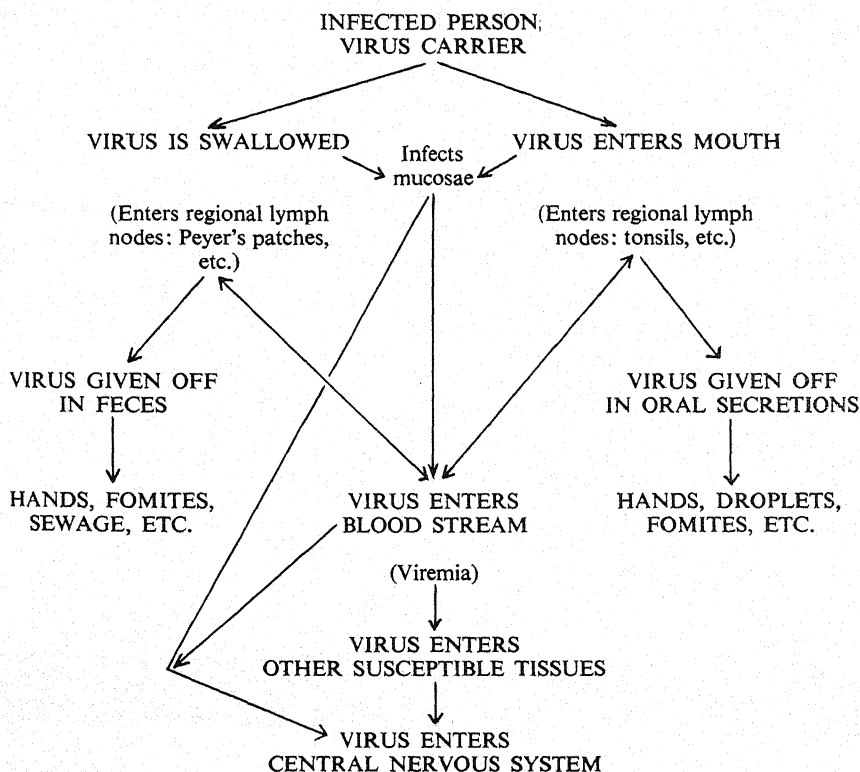


Fig. 45-3. One theory as to the course of poliomyelitic infection in the body, its portals of entry and exit and its principal means of transmission. (Adapted from Bodian, D., *Science*, 1955, vol. 122; and Sabin, A. B., *Science*, 1956, vol. 123.)

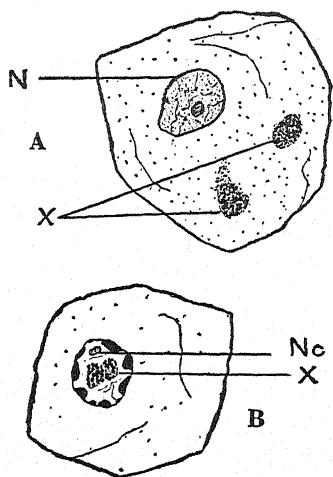


Fig. 45-4. A, one type of cytoplasmic viral inclusion bodies is shown at X; nucleus at N. B, intranuclear inclusions in liver cell of victim of yellow fever. Nc, nucleolus; X, inclusion bodies. Note the characteristic lobulations at the nuclear membrane. (Redrawn from Cowdry and Kitchen.)

tract to the central nervous system. It is due to invasion of the central nervous system by the virus that the paralytic and lethal bulbar effects of polio arise. Thus the principal value of the vaccine is to lower the incidence of paralytic and fatal polio. The rates of nonparalytic polio in vaccinated and unvaccinated groups are not significantly different.

The vaccine offers good protection against virus types II and III, good to fair protection against type I virus.

It appears that the Salk vaccine is an enormously valuable weapon against polio. More effective vaccines are being studied by Salk and others, especially vaccines made with *live, attenuated* polio virus.

**Rabies.** This disease has been discussed in the chapter on immunity since Pasteur's studies on rabies vaccination are of historical immunological interest. Almost every species of mammal is susceptible, including vampire and insectivorous bats. The virus occurs in saliva of infected animals and is transmitted by their bite. It invades the cells of the central nervous system and causes the appearance, in their cytoplasm (and sometimes nucleus), of certain distinctive granules\* called *Negri bodies* after the Italian scientist who first described them in 1903. This invasion results in the clinical picture of rabies. By examination of smears or sections of the brain of rabid animals stained by the method of Sellers, the Negri bodies can usually be seen, easily recognized, and used as the basis for diagnosis. A more reliable but more time-consuming means of diagnosis is to inject saliva or brain tissue of rabid animals or persons into the brains of normal mice, guinea pigs or other animals. These develop the typical disease. Specific immune serum can be used to protect control animals, thus confirming the diagnosis.

**CARE OF RABID OR SUSPECT ANIMALS.** A dog which bites a person should not be killed but confined and kept under observation for at least ten days.

\* Similar granules are seen in many tissue cells infected by viruses. Each type of granule is more or less distinctive of the infecting virus. According to their location or discoverer the granules are spoken of as *intranuclear inclusion bodies* or *cytoplasmic inclusion bodies*, Councilman bodies (yellow fever), etc. (Fig. 45-4).

If rabid, it will die after exhibiting definite symptoms which include irritability and restlessness, anxiety, fear and failure to recognize its master; usually followed by paralysis of the throat and inability to eat or drink, drooling of saliva ("foaming at the mouth"), and finally general paralysis and death. If not confined it has a tendency to wander long distances and to bite various objects, animals and persons ("furious rabies") unless paralysis sets in earlier when it may merely skulk or run about dumbly ("dumb rabies"). In cats the furious stage is terrifying to observe and the animal is exceedingly dangerous because it covers itself with saliva and the slightest scratch of tooth or claw can infect. The cat will attack on sight. Even objects wetted with the saliva of a rabid animal can infect.

The head of an animal suspected to have died with rabies should be removed, preferably by a veterinarian, and sent *immediately* (*packed in a bucket of ice or "dry ice"*) to the local or State Health Department for examination.

**CARE OF BITTEN PERSONS.** Any person bitten by a suspected animal should consult a physician immediately. The wound should be given immediate care by thorough washing of the interior of the wound with medicinal soap and water. Pasteur treatment should be used only in cases in which *effective* exposure to a *rabid* animal is reasonably sure. If the treatment is to be used, it should be instituted at once. If the animal is under observation and shows no symptoms within 8 days, the treatment may be discontinued. Some authorities delay treatment during the observation period as the series of injections is unpleasant at best and may have serious results, presumably due to allergy to rabbit-brain tissue. If the animal is not caught, the treatment should be completed. The use of immediate (72 hours) passive protection with immune serum is of recognized value in case of very suspicious or severe bites.

**CONTROL OF RABIES.** The control of rabies (since no cure is known) consists in vaccination and licensing of dogs and cats, destruction of strays, quarantine of dogs and cats when the disease is prevalent, and general measures to prevent spread among wild animals. Excellent new vaccines containing living rabies virus attenuated by passage in chick embryos ("Avianized" vaccine, Flury strain) are now available for use in animals. They may also prove satisfactory for use in man but are not yet generally accepted for human use.

### ENTERIC VIRUSES

It has been made clear that the poliomyelitis viruses, though generally spoken of as neurotropic, are really primarily enteric.

Another group of viruses often found in the intestines and causing polio-like and/or flu-like symptoms comprises the Cocksackie viruses\* or "C" viruses. Numerous other viral agents sometimes occur in feces; influenza, adenoviruses, hepatitis, mumps, and others.

There is a disease (or group of diseases) called *epidemic nausea and vomiting*, *infantile diarrhea* or *infantile gastroenteritis*, presumably due to one or more viruses. The viral agents causing it are still not fully identified but are readily transmitted, by feces presumably, and may well be the cause of death in infants and much of our adult gastrointestinal woes (gastrointestinal "flu,"

\* Named for the town of Cocksackie, New York, where they were first discovered.

etc.). A group of related viruses are often referred to as the human enteric (HE) viruses. A group of similar enteric viruses, of still imperfectly known significance, was originally called "Orphan" viruses, because they were unidentified and unnamed. In tissue culture they cause visible degenerative changes and/or death in the tissue cells of the culture. They are said to be *cyto-pathogenic*. This is suggestive evidence of pathogenicity. These two groups (HE and Orphan) are now collectively spoken of as the ECHO (Enteric Cytopathogenic Human Orphan) viruses. The exact role of these cytopathogenic agents in human disease remains to be clarified.

### NEOPLASTIC DISEASES

One of the great mysteries of human pathology is the etiology and nature of "cancer." There is a great variety of cancers (malignant tumors), or, more properly, neoplasms of both plants and animals, and there is a variety of causative agents. Whether any of these agents is a living organism is still unknown with regard to human neoplasms, but several neoplasms of animals are due to viruses. Some plant neoplasms (e.g., crown gall) are due to bacteria, some (e.g., "wound tumor") are due to viruses. Whether, by studies of the neoplasms of animals or plants, the secret of human neoplasms may eventually be revealed, none can say. There are many suggestive analogies between the infectious animal and vegetable neoplasms on the one hand and human neoplasms on the other but the actual relationship, if any, is still obscure.

**Neoplasms, Viruses and Mutation.** The cause of human malignant neoplasms is unknown. There may be various causative agents. We do know, however, that the development of some neoplasms in human beings has long been associated with mechanical irritation or injury. In the presence of irritation and injury, cells are killed and new growth tends to replace them. In the continued presence of the irritating and injurious agent it is readily conceivable that the cells of the new growth may be altered\* so that they mutate, grow awry, and become malignant, independently-growing cells. X-rays, ultraviolet and other irradiations, certain coal-tar derivatives (methyl cholanthrene, etc.), mustard oils, petroleum oils, tar, pitch, certain aromatic amines and other irritating and injurious agents have all been shown to induce neoplasms. They are said to be *carcinogenic*. How these agents act on living cells is not certain but one striking effect of all of them is the production of mutations in various cells (including sex cells): microorganisms, plants, insects, animals. Such agents are not only carcinogenic but mutagenic.

Neoplasms thus appear to begin, at least in some instances, as mutated or infected cells which act as though foreign to the tissues and body of origin; are no longer held in check by the normal growth-regulating hormones, etc. They grow independently, often without regard for normal physiological limitations, spreading and growing everywhere, each tumor cell a malignant parasite. Sometimes they grow slowly and within definite bounds (benign tumors). No connection between human neoplasms and viruses has been finally established, but it is clear in animals and plants.

It has been suggested, on reasonable grounds, that the true role of car-

\* Infected with some neoplastic viral agent?

cinogenic agents is to *induce* cancer viruses which may, like phage in lyso-genic bacteria, be latent in the tissue cells of certain people. Any reader who solves this problem will be recommended for the Nobel prize!

**Antagonisms of Viruses and Neoplasms.** One of the most interesting developments concerning the relationship between viruses and neoplasms is the observation that certain viruses not only inhibit the growth of certain viral neoplasms of animals but actually cause them to regress and disappear, leaving behind a solid immunity to the neoplastic disease involved. The most active viruses in attacking viral neoplasms are certain of the arthropod-borne, neurotropic viruses—Russian-Spring-Summer (R-S-S) encephalitis, louping-ill, West Nile encephalitis, Japanese B encephalitis, St. Louis encephalitis, Semliki Forest encephalitis. Some of these have the power to inhibit or destroy sarcoma 180 (an infectious neoplasm of mice) and/or infectious lymphoid neoplasm (RPL-12) of chickens and/or infectious rabbit fibroma. Note that these neoplasms are all transmissible, i.e., caused by virus-like agents. The antagonistic neurotropic viruses are effective whether inoculated directly into the neoplasm or at a distant site. The neurotropic viruses in some instances seem to have a special affinity for the neoplasm tissues and to infect, grow in, and destroy them.

It is of especial interest that several human cancers in tissue culture have been found susceptible to one or more of some common viruses: polio, herpes, vaccinia, Coxsackie, adeno, and others. What is true of these cancers is very likely to be true of other cancers, i.e., that they are vulnerable to viral attack.

The significance of these observations in relation to the possibility of developing "neoplastotrophic" strains of virus for the cure of "cancer" should serve as a stimulus to any student of microbiology.

#### REFERENCES

- Bawden, F. C.: *Plant Viruses and Virus Diseases*. 3rd ed. The Chronica Botanica Co., Waltham, Mass., 1950.
- Biology of Poliomyelitis. *Ann. New York Acad. Sci.*, 1955, 61:737.
- Brown, G. C., and Smith, D. C.: Serologic response of infants and preschool children to poliomyelitis vaccine. *J.A.M.A.*, 1956, 167:399.
- Burnet, F. M.: *Principles of Animal Virology*. Academic Press, Inc., New York, 1955.
- Dalldorf, G.: *Introduction to Virology*. Charles C Thomas, Springfield, Ill., 1955.
- Dalldorf, G.: The Coxsackie viruses. *Ann. Rev. Microbiol.*, 1955, 9:277.
- Debre, R., et al.: Poliomyelitis. W. H. O. Monograph Series No. 26, 1955. Columbia University Press, New York 27, N. Y.
- Downie, A. W., and Dumbell, K. R.: Pox viruses. *Ann. Rev. Microbiol.*, 1956, 10:237.
- Editorial: Ornithosis. *J.A.M.A.*, 1956, 160:1057.
- Editorial: The adenoviruses. *J.A.M.A.*, 1957, 163:40.
- Elton, N. W.: Yellow fever in Central America: the imminent threat to Mexico and the United States. *Am. J. Pub. Health*, 1956, 46:1259.
- Enright, J. B.: Bats and their relation to rabies. *Ann. Rev. Microbiol.*, 1956, 10:369.
- Expert Committee on Influenza (First Report), 1953. World Health Organization Technical Reports Series No. 64. Columbia University Press, New York 27, N. Y.
- Francis, T., Jr. (Chairman): *Diagnostic Procedures for Virus and Rickettsial Diseases*. 2nd ed. Am. Pub. Health Assoc., New York 19, N. Y., 1956.
- Gray, G. W.: Unknown viruses. *Sci. Am.*, 1955, 192:60.
- Gross, L.: Viral etiology of cancer and leukemia? *J.A.M.A.*, 1956, 162:1318.
- Havens, W. P., Jr.: Hepatitis, yellow fever and dengue. *Ann. Rev. Microbiol.*, 1954, 8:289.
- Hilleman, M. R., and others: Adenovirus (RI-APC-ARD) vaccine for prevention of acute respiratory illness: I, II. *J.A.M.A.*, 1957, 163:4, 9.

- Karzon, D. T., Barron, A. L., and Winkelstein, W., Jr.: Isolation of ECHO virus type 6 during outbreak of seasonal aseptic meningitis. *J.A.M.A.*, 1956, 162:1298.
- Koprowski, H., et al.: Immunization of infants with living attenuated poliomyelitis virus. *J.A.M.A.*, 1956, 162:1281.
- Krugman, S., and Ward, R.: The rubella problem: Clinical aspects, risk of fetal abnormality and method of prevention. *J. Pediat.*, 1954, 44:489.
- Luria, S. E.: General Virology. John Wiley and Sons, Inc., New York, 1953.
- Moore, A. E.: Effects of viruses on tumors. *Ann. Rev. Microbiol.*, 1954, 8:393.
- Rabies. World Health Organization Technical Report Series No. 82. Columbia University Press, New York 27, N. Y., 1954.
- Rhodes, A. J., and Van Rooyen, C. E.: Textbook of Virology. 2nd ed. The Williams & Wilkins Co., Baltimore, 1953.
- Rivers, T. M., et al.: Viral and Rickettsial Infections of Man. 2nd ed. J. B. Lippincott Co., Philadelphia, 1952.
- Sabin, A. B.: Present status of attenuated live-virus poliomyelitis vaccine. *J.A.M.A.*, 1956, 162:1589.
- Salk, J. E.: Poliomyelitis vaccination in the fall of 1956. *Am. J. Pub. Health*, 1957, 47:1.
- Strode, G. K., Bugher, J. C., Kerr, J. A., Smith, H. H., Smithburn, K. C., Taylor, R. M., Theiler, M., Warren, A. J., and Whitman, L.: Yellow Fever. McGraw-Hill Book Co., New York, 1951.
- Tierkel, E. S.: The present status of rabies control. *Am. J. Pub. Health*, 1955, 45:677.
- Various Authors: Viruses as causative agents in cancer. *Ann. New York Acad. Sci.*, 1952, 54 (Art. 6): 869.
- Various Authors, Dynamics of Virus and Rickettsial Infections. The Blakiston Co., New York, 1954.

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